

Full Length Research Paper

Molecular diagnosis of vaginal candidiasis by polymerase chain reaction (PCR) and random amplification polymorphism DNA (RAPD-PCR) in Babylon Province, Iraq

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Most women suffer from severe vaginal candidiasis caused by *Candida* spp. Vaginal infections represent a second type of common disease. This infection occurs commonly in married and pregnant women and immunocompromised patients. This study aimed to survey, isolate and diagnose the vaginal *Candida* spp. using phenotypic and molecular assays. *Candida* isolates were recovered from patients attending main hospital in Babylon Province, Iraq from July 2011 to April 2012. Samples were subcultured on CHROMagar for preliminary identification of *Candida* isolates. Germ tube and chlamyospore formation were also performed to confirm identification. DNA of representative isolates was extracted for polymerase chain reaction (PCR) assays and analyzed using gel image patterns of bands for different isolates based on ultraviolet transilluminator band software. Results show that CHROMagar was a good tool for preliminary identification of *Candida* isolates. Molecular identification of PCR and random amplification polymorphism DNA (RAPD-PCR) assays were used to group the representative *Candida* isolates into different genetic patterns; phylogenetic tree was generated based on the information available in gel images. This study concludes that PCR and RAPD PCR assays confirmed more the diagnosis of *Candida* isolates than culture on CHROMagar medium and other phenotypic tests.

Key words: Vaginal, *Candida*, diagnosis, CHROMagar, polymerase chain reaction (PCR), random amplification polymorphism DNA (RAPD)-PCR, phylogenetic tree.

INTRODUCTION

More than 70% of women are exposed to vaginal candidiasis caused by a group of *Candida* spp. once or twice a year or at least once in their lifetime (Corsello et al., 2003). The clinical symptoms include itching, edema, erythema of the vulva, vaginal discharge of white clots and pain. Paulitssch et al. (2006.) showed that *Candida albicans* is responsible for 70-90% of all vaginal candidiasis in Austria while many other studies showed that *Candida glabrata* has been the most frequent pathogen that causes vaginal Candidiasis (Novikova et al., 2002; Lopes

Consolaro et al., 2004). Ozcan et al. (2006) reported that *C. glabrata* is responsible for 14% of infections in immune competent women. *Candida* is a member of normal flora in vagina and oral cavity; however, there are many predisposing factors that can lead to these infections such as pregnancy, long term use of antibiotics, diabetes mellitus, using of corticosteroids, HIV infection, and being immunocompromised (Fidel and Sobel, 1996; Duerr et al., 1997, 2003; Corsello et al., 2003; Beltramene et al., 2006).

The routine diagnosis and identification techniques of *Candida* spp. can be performed by conventional culture techniques followed by biochemical tests, but most of these tests are laborious, time consuming, and do not give clear cut results (Hospenthal et al., 2006). On the other hand, biochemical tests sometime may fail to identify *Candida* spp. (Goswami et al., 2006.).

Molecular tests are much more rapid, and have higher sensitivity and specificity as compared to conventional techniques. Rapid identification is important for treating infections caused by *Candida* species especially vaginal infections that cause unbearable itching and yellow exudation of white clots (Fidel and Sobel, 1996). Rapid diagnosis by polymerase chain reaction (PCR) and random amplification polymorphism DNA (RAPD) assays facilitate patients' treatment with vaginal antifungal antibiotics (Jordan and Durso, 1996; Ray et al., 2007). The simple PCR detects genetic variation but sometimes not clear enough for typing all *Candida* spp. For this reason, a RAPD technique is required for genetic typing of *Candida* spp. (Mannarelli and Kurtzman, 1998). There are highly risky vaginal candidiasis that could sometime lead to women's sterility by increasing the agglutination of sperms and reducing sperm motility (WHO, 2001). This study aims to survey and diagnose vaginal candidiasis using phenotypic and PCR techniques to detect the different species of *Candida* involved.

MATERIALS AND METHODS

Sampling

One hundred and five (105) vaginal samples were collected from married patients (16-45 years old) attending the outpatient clinics in the main hospital of Babylon Province, Iraq from July 2011 to April 2012. All samples were collected by sterile vaginal swabs during a gynecological examination. Samples were directly immersed in Sabouraud agar medium (SDA) and incubated for 24-48 h at 30°C.

Germ tube test

A loopful of yeast cells suspension was inoculated into 0.5 ml of human serum and incubated at 37°C for 3 h. After incubation period, it was examined under field microscope. Germ tube was considered as a lateral tube without septum and had no constriction at initiating site, which is a positive test for *C. albicans* (Marinho et al., 2010).

Chlamyospore formation test

Chlamyospore formation test was fulfilled by pick up. Inoculums of yeast colonies were subcultured on rice agar medium (10 g rice polish and 15 g agar all dissolved in 1 L of distilled water) and incubated at 30°C for 72 h. Then they were mounted by adhesive tape on slide with lactophenol cotton blue.

CHROMagar culture

The purified single colonies on SDA were streaked on Chromoagr and incubated for 24-48 h. *Candida* isolates were classified

according to their colors on Chromoagr based on colored key designed by Campbell et al. (1998) and Nadeem et al. (2010).

Extraction of DNA

Twelve isolates from each color group were selected randomly as a representative of all *Candida* isolated in this study for DNA extraction and PCR assays. DNA of *Candida* spp. was extracted by picking single colony using sterile loop and suspended into 300 µl of lysis buffer (10 mM Tris, 1mMEDTA (pH8), 1% SDS, 100 mM NaCl, 2% Tween 80), 300 µl phenol-chloroform (1:1); it was shaken for 5 min and centrifuged at 1000 rpm. The supernatant was transferred to new tube and equal volume of chloroform was added, mixed and centrifuged. 500 µl ethanol was added to the supernatant and centrifuged at 10000 rpm for 7 min. Dry DNA pellet was re-suspended in 100 TE buffer and stored at -20°C until use (Mousavi et al., 2007).

PCR assay

The universal primer pair that targets sequences site of the 18S rRNA gene of *Candida* spp. was used as described by Metwally et al. (2008). This primer pair included CANIA (5'-GAGGGCAAGTCTGGTG-3') and CANIB (5'-CTGCTTTGAACACTCTAA-3').

A specific primer pair was used for diagnosis of *C. albicans* based on the sequence data for (ITS) region, CALB1: (5'-TTTATCAACTTGTGTACACCAGA-3'), and CALB2: (5'-ATCCCGCCTTACCCTACCG-3') (Luo and Mitchell, 2002). The PCR mixture (25 µl) consisted of 5 µl of 20x Master Mix (Promega), 2 µl (10 pmole) of each primer and 1 µl template DNA, made up to 25 µl with molecular-grade water. The PCR mixture was amplified by thermal cycler PCR System (Labnet, USA) using the following conditions: first denaturized temperature of 95 for 5 min followed by 30 cycles including initial denaturation temperature of 95 for 30 s; annealing temperature (56°C) for 1.5 min; extensions temperature, 72°C for 1 min, and final extension temperature, 72°C for 10 min. The PCR products were run on 1.2% agarose gel (Bio Basic Canada Inc.) and electrophoreses were performed at 100 V in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator.

RAPD-PCR

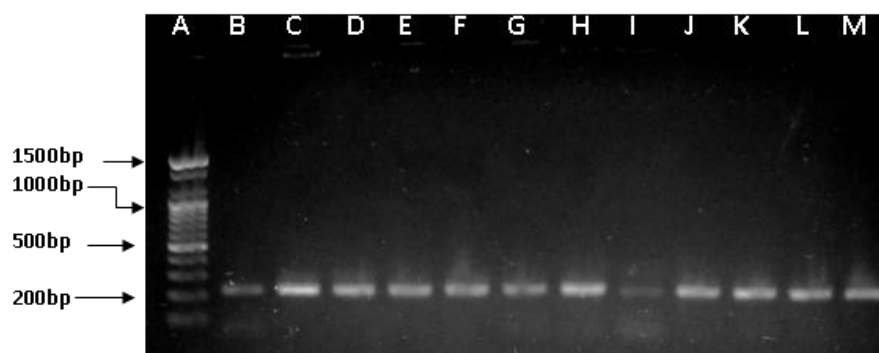
RAPD-PCR was performed with total volume of 50 µl consisting of 10 µl of 50x master mix (Promiga, USA) with 37 µl molecular-grade water, 0.5 µl template DNA and 1.5 µl (50 pmole) for each random primers RP1-4: TAGGATCAGA and SOY: AGGTCCTGA (Valerio et al., 2006). Amplified PCR conditions were as follows: 95°C for 4 min, 38 cycle 94°C for 1 min, 36°C for 1.5 min, 72°C for 1.5 min, 72°C for 8 min. The PCR products were run on 1.8% agarose gel (Bio Basic Canada Inc) and electrophoreses were performed at 60 V in TBE buffer. Gel was stained with 0.05% ethidium bromide mixed with agarose. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator.

Phylogenetic analysis

Phylogenetic tree of RAPD-PCR products for *Candida* isolates was created by clustering methods applied on distance matrix unweighted pair group method with arithmetic mean (UPGM) which offers automatic lane/band detection, band matching, molecular weight computation. The phylogeny tree computation was analyzed based

Table 1. Germ tube, chlamyospore formation and colony color of vaginal *Candida* spp.

Species	No. of isolates	Germ tube		Chlamyospore		Colony color on CHROMagar and texture
		No (%)	No (%)	No. (%)	No. (%)	
<i>C. albicans</i>	83	78	94	80	96.4	Light green and smooth
<i>C. tropicalis</i>	3	0	0	0	0	Blue-pink
<i>C. glabrata</i>	20	0	0	0	0	Pink to cream
<i>C. krusei</i>	9	0	0	0	0	White-pink with white border
<i>C. parapsilosis</i>	4	0	0	0	0	White cream
<i>C. dubliniensis</i>	3	2	66.6	0	0	Dark green and rough

**Figure 1.** Agarose gel electrophoresis of amplified PCR products for *Candida* isolates by universal primer CANIA and CANIB, Lane A 100 bp. molecular size marker, lanes B-M represent *Candida* isolates.

on UVI band software GD/45230 for gel image analysis. The software is able to analyze gel image patterns of bands for different isolates or species and generates phylogenetic tree based on the information available in gel image; it evaluated the similarity coefficient factor according to Mackenstedt et al. (1994).

RESULTS AND DISCUSSION

Germ tube and chlamyospore formation

Out of 105 vaginal samples, only ninety seven revealed positive vaginal candidiasis, of which 122 isolates of *Candida* were recovered. Most *C. albicans* isolates formed germ tubes and chlamyospores on Rice agar (Table 1). Results show that 94 and 96.4% of *C. albicans* isolates formed germ tubes and chlamyospores, respectively. These results are in line with those of Beheshti et al. (1975).

Identification of *Candida* isolates

Out of 122 *Candida* isolates of vaginal candidiasis, 83 that appeared as light green and smooth colonies were diagnosed as *C. albicans*, while the rest belonged to six other species of *Candida* which are *Candida tropicalis*, *C. glabrata*, *Candida krusei*, *Candida parapsilosis* and

Candida dubliniensis based on the description of Campbell et al. (1998) and Nadeem et al. (2010). The results showed that chromoagar contributed to differentiation of *Candida* spp. into two groups, *C. albicans* and non-*albicans* species. However, this medium was unable to differentiate *C. albicans* from *C. dubliniensis* and *C. tropicalis* because there was no clear borderline between these yeasts due to their colors on CHROMagar (Beighoton et al., 1995; Nadeem et al., 2010).

Molecular diagnosis by PCR

Universal primer pair CANIA and CANIB were successful in amplification of target region of 18S rRNA gene for *Candida* spp. and in production of approximately 210 bp (Figure 1). Regarding specific primer pair for *C. albicans* CALB1 and CALB2, it yielded approximately amplicon size of 273-280 bp, but it did not yield amplification products with non-*albicans* species like lane B and lane I (Figure 2). The amplicon sizes of this study are same with the results of previous studies that CANIA and CANIB generated amplicon size of 210 bp while CALB1 and CALB2 produced amplicon size of approximately 273 bp. These results are in line with that of Luo and Mitchell (2002) and Metwally et al. (2008). The PCR results conform to the phenotypic patterns in

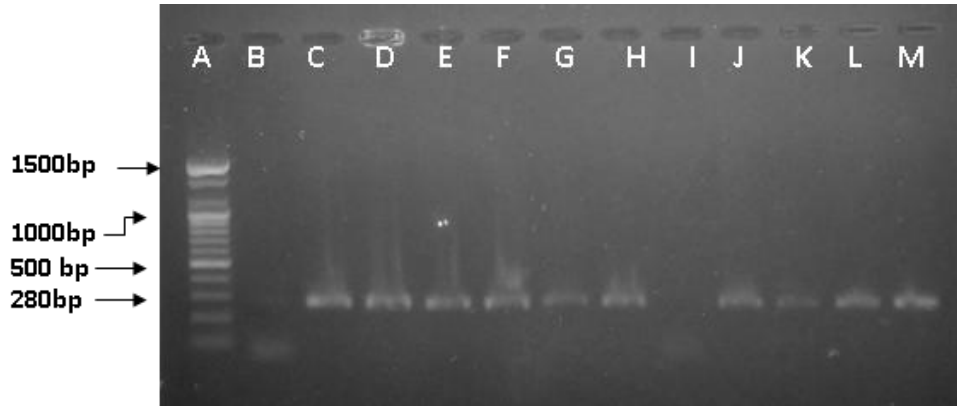


Figure 2. Agarose gel electrophoresis of amplified PCR products for *Candida* isolates by specific primer CAIB1 and CALB2, Lane A 100 bp molecular size marker, lanes C-H, J-M represent *C. albicans*, lane B and lane I non-albicans isolates.

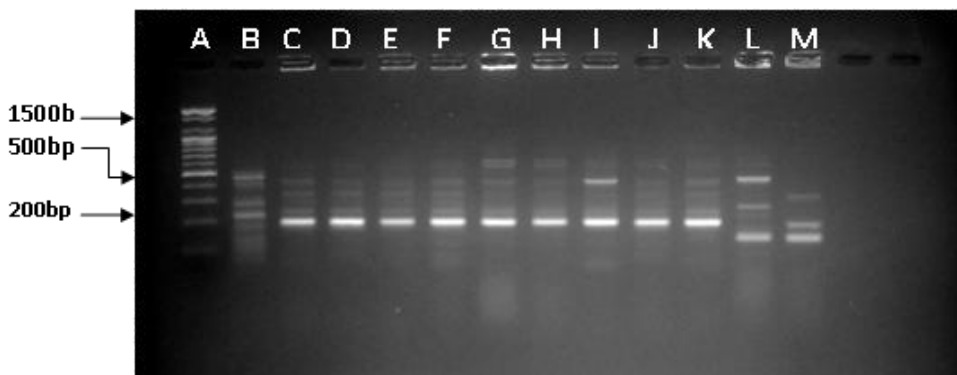


Figure 3. Agarose gel electrophoresis of amplified RAPD-PCR products for 12 clinical *Candida* isolates using primer SOY: Lane A size marker 100 bp; lanes B-M, isolates of *Candida*.

Figure 1; the specific primers do not amplify the target DNA of non-albican isolates; at the same time, these specific primers amplified the target DNA of *C. tropicalis* which revealed a blue color. This type of contradictory diagnosis by CHROMagar when compared with molecular diagnosis may reveal that CHROMagar is not always essential for presumptive diagnosis of *Candida* species. So there was required a combination of some phenotypic and molecular methods for presumptive identification of most *Candida* isolates. These results are same with the ones obtained by Ahmed et al. (2002) who stated the limitation of phenotypic tests and the molecular methods, especially PCR which is being increasingly used for rapid detection of *Candida* than conventional phenotypic methods.

Molecular diagnosis by RAPD-PCR

Twelve isolates of *Candida* detected by RAPD analysis with two primers, SOY and RP1-4 revealed homogenous RAPD fingerprinting patterns for *Candida* isolates (lanes C-K); for both primers, others were heterogeneous RAPD

fingerprinting as in lanes B, L and M. Typing with these primers showed clear characteristic RAPD fingerprinting patterns for each isolate. The band patterns obtained by SOY primer were highly different, which helps in proper typing of most isolates; for instance, isolates C,D,G,H, and K revealed unique bands belonging to *C. albicans*, while the isolates E, F, and J also belonged to *C. albicans* but with some variations (Figure 3).

The band patterns obtained by RP1-4 primer classified eight isolates (lanes B-G, I and J) of *C. albicans* into one genotype with few variations (Figure 5). While the isolate of *C. glabrata* (lane B) was classified into unique genotype. However, the last three isolates (lanes L-M) showed distinctive genotypes (Figure 5). These results are same with the report of Valério et al. (2006) who found that RAPD-PCR profiles of *Candida* isolates using RP1-4 and SOY primers were able to characterize interspecific polymorphisms among *Candida* isolates. They concluded that there were no polymorphic traits among *C. albicans* isolates and referred to some variations in RAPD-PCR profiles among isolates of the same species. They also mentioned that these molecular methods provided valuable

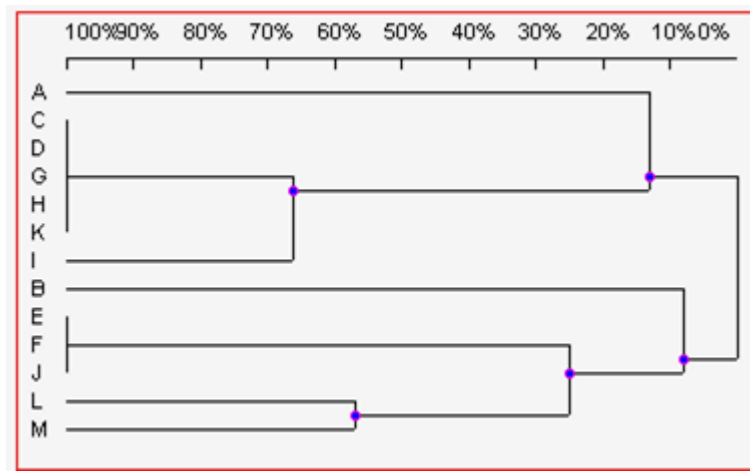


Figure 4. Phylogenetic relationships (homologous coefficient (%)) between different isolates of *Candida*. The consensus tree was based on data of RAPD-PCR by SOY primer generated via UPGMA cluster analysis. A, Molecular size marker 100 bp; C-M, clinical isolates of *Candida*.

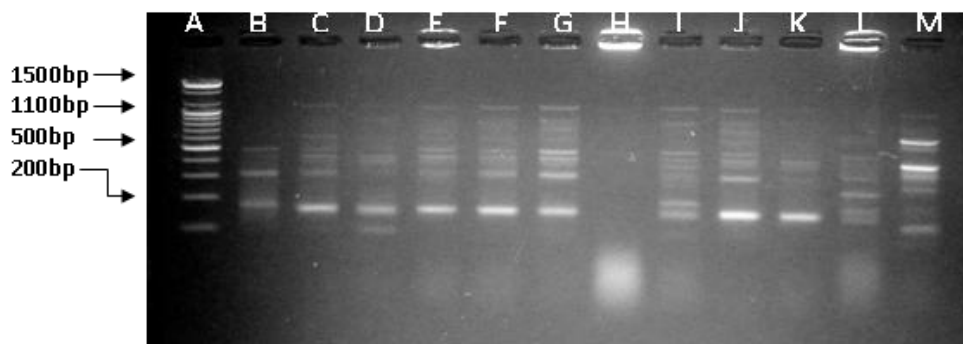


Figure 5. RAPD-PCR patterns of *Candida* isolates by primers RP1-4. Lane A is 100 bp ladder molecular size marker, lanes B-M *Candida* isolates.

information for patients' management as compared to the traditional methods of identification of *Candida* spp.

Phylogenetic analysis data of RAPD-PCR

Phylogenetic diversity among different *Candida* isolates was determined by converting RAPD-PCR data of two primers, SOY and RP1-4 into similarity matrix and analyzed by UPGMA to produce a phylogenetic tree. The bands pattern obtained by SOY primer varied and all isolates were classified completely into three major groups based on homology coefficient percentage: first one included subgroup of five isolates (C, D, G, H and K) which had 100% homology and isolate I which had 66% homology with previous subgroup. The second major group included the isolates (E, F and D, J) with 100% homology and two isolates (L, M) with 60% homology;

while the third group was represented by one isolate (B) (Figure 4). Valério et al. (2006) also showed no polymorphism in some *C. albicans* isolates and reported some variation in the RAPD-PCR profiles between isolates in the same species.

Phylogenetic tree patterns obtained by RP1-4 primer varied. All isolates were typed into four major groups based on homology coefficient percentage: first one included subgroup of four isolates (B, C, and E) which have 80-100% homology, second group included L, M, N, and O isolates, third group (D, F, H, G, J and K) had 25-84% homology and the fourth group was represented by 1 isolate which revealed 10% homology with other groups (Figure 6). The results show that RAPD-PCR patterns had rich and diversity of DNA banding patterns that allowed the typing of 12 isolates at isolates level. In fact, although the isolates were highly homologous using simple PCR to produce monomorphic bands for isolates

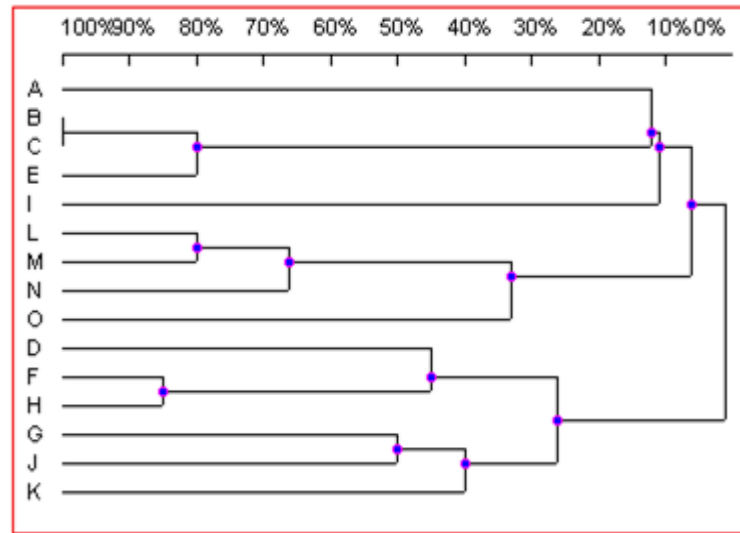


Figure 6. Phylogenetic relationships (homologous coefficient (%)) between different isolates of *Candida*. The consensus tree were based on data of RAPD-PCR by RP1-4 primer generated via UPGMA cluster analysis. A-Molecular marker 100 bp, B-K clinical isolates of *Candida* isolates.

of *Candida* (Figure 2). But, RAPD-PCR patterns revealed polymorphisms in these monomorphic bands and led to the creation of many major group of typing. These results are in agreement with those of Tamura et al. (2001).

With this study, we conclude that RAPD-PCR assay can be used as diagnostic and differentiating tool for *Candida* spp. in clinical laboratories. The results of PCR and RAPD-PCR procedures described here allowed the identification of *C. albicans* in less than 1 day, using a simple technique to obtain DNA when compared with phenotypic tests that consume more time and cost.

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