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**RESEARCH ARTICLE**

# RAPID IDENTIFICATION OF NON-*CANDIDA ALBICANS CANDIDA* (NCAC) SPECIES ISOLATED FROM FEMALE PATIENTS CLINICALLY DIAGNOSED WITH VULVOVAGINITIS BY USING MULTIPLEX PCR

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**ABSTRACT**

**Background:** The majority of women with *Candida* vaginitis suffer from uncomplicated vaginitis characterized by sporadic attacks of mild to moderate severity due to *C. albicans*, and these attacks occur in healthy adult women without any predisposing factors. In contrast, approximately 10% of women suffer from complicated *Candida* vaginitis, in which attacks either are more severe, occur on a recurrent basis, or are due to non-*albicans Candida* species. Although several conventional methods are routinely used for determintion; however, *Candida* vaginitis or vulvovaginitis a challenge for patients and gynecologists.

**Aims and objectives:** Aims of the current study were to find out the prevalence of candidial vulvovaginitis attributed due to Non-*Candida albicans Candida* (NCAC) species such as *Candida glabrata, Candida tropiclis* and *Candida parapsilosis*, and to use a multiplex PCR technique as a rapid identification method for these Non-*Candida albicans Candida* species isolated from female patients clinically diagnosed with vulvovaginitis.

**Materials and Methods:** In this study vaginal swabs from 160 female patients were used for culture, Api Candida system, VITEK 2 system and multiplex PCR analysis. Multiplex PCR was performed with species-specific primers targeted to the ITS region of rRNA gene of *C. glabrata, C. tropicalis* and *C. parapsilosis*. The result of the multiplex PCR was compared with conventional culture, Api Candida system and VITEK 2 system methods. The positive multiplex PCR product was identified by presence of ~ 423 bp, ~357 bp and ~300 bp amplicons of ITS region of rRNA gene for the *C. glabrata, C. tropicalis* and *C. parapsilosis*, reaspectively.

**Results:** Conventional methods of candidial culture, Api Candida system and VITEK 2 system, showed that to sum up 100 out of 160 vaginal swabs were detected for *Candida* species; To sum up 16 out of 100 vaginal swabs were detected for *C. glabrata*, 10 out of 100 vaginal swabs were detected for *C. tropicalis,* 6 out of 100 vaginal swabs were detected for *C. parapsilosis* and 37 out of 100 vaginal swabs were detected for other *Candida* species. Five of the 60 samples that were negative by conventional methods were positive by multiplex PCR. Statistical analysis revealed that the PCR to have a sensitivity of 95.5 % in the detection of Non-*Candida albicans* in vulvovaginitis.

**Conclusion:** This multiplex PCR method provides a sensitive, rapid, and reliable alternative to conventional methods to identify *Non-Candida albicans Candida* species isolated from female patients clinically diagnosed with vulvovaginitis.



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## INTRODUCTION

In last year there has been a significant increase in the incidence of fungal infections in humans (Lass-Florl, 2009). Such infections may either be superficial, affecting the skin, hair, nails and mucosal membranes, or systemic, involving major body organs (Ruping *et al*., 2008). A number of factors have been implicated in this increased occurrence of fungal disease, but it is generally accepted that the increased and widespread use of certain medical practices, such as immunosuppressive therapies, invasive surgical procedures and use of broad-spectrum antibiotics are significant (Hagerty *et al*., 2003 and Kojic, 2004). Of the fungi regarded as human pathogens, the members of the genus *Candida* are the most frequently recovered from human fungal infection. The *Candida* genus contains over 150 heterogeneous species, but only a minority has been implicated in human candidosis. Additionally, it is known that approximately 65% of *Candida species* are unable to grow at a temperature of 37 ºC, which precludes these species from being successful pathogens or

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indeed commensals of humans (Calderone, 2002). Of the *Candida species* isolated from humans, *Candida albicans* is the most prevalent under both healthy and disease (Samaranayake *et al*., 2002) conditions. However, while mycological studies have shown that *C. albicans* represents over 80% of isolates from all forms of human candidosis in the last two decades, the number of infections due to Non-*Candida albicans Candida* (NCAC) species such as *C. glabrata, C. tropiclis* and *C. parapsilosis* has increased significantly (Kauffman *et al*., 2000; Manzano-Gayosso *et al*., 2008 and Ruan, S. and Hsueh, 2009). The apparent increased involvement of NCAC species in human candidosis may partly be related to improvements in diagnostic methods, such as the use of chromogenic media with the ability to differentiate *Candida species*, as well as the introduction of molecular techniques in the routine diagnosis of fungemia (Liguori, 2009). However, the high prevalence of NCAC species in disease could also be a reflection of their inherently higher level of resistance to certain antifungal drugs compared with *C. albicans*, as this would promote their persistence, possibly to the detriment of *C. albicans*, in mixed species infections treated with traditional antifungal agents (Gonzalez, 2008). The pathogenicity of *Candida species* is attributed to certain virulence factors, such as the ability to evade host defenses, adherence, biofilm formation (on host tissue and on medical devices) and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin (Silva *et al*., 2011). An increase in the number of yeasts that are resistant to antifungal drugs is recognized worldwide; therefore, the use of in vitro laboratory tests may aid the doctor in choosing an appropriate therapy (Ingham, *et al*., 2012). The ability of *Candida species* to form drug-resistant biofilms is an important factor in their contribution to human disease (Rajendran *et al*., 2010).

## MATERIALS AND METHODS

### Samples collection

Samples were obtained from female patients clinically diagnosed with vulvovaginitis in four hospitals at Baghdad which are; Baghdad Teaching Hospital- Medical city, Al-Karama Teaching Hospital, Al-Kadhmiya General Teachining Hospital, Al-Yarmuk General Teaching Hospital, and from private gynecologist clinics in Al-Aziziyah city, Kut governorate, Iraq for a period of 3 months. One hundred sixty cases of vulvovaginitis were investigated in this study. Patients' ages ranged from 20 to 40 years of age. This study was carried out after obtaining the approval from the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ Baghdad University and Ministry of Health/ Iraq. Vaginal swabs were obtained from 160 female patients clinically diagnosed with vulvovaginitis by gynecologist with a sterile swabs and cultured on Sabouraud's dextrose agar plus chloramphenicol (SC) (Merck, Germany) at 37°C for 48 hours under aerobic condition, then the positive cultures of *Candida* species on Sabouraud's dextrose agar plus chloramphenicol were cultured on chromID Candida agar (Biomerieux, France) at 37°C for 48 hours under aerobic condition also, as deferential medium for Non-*Candida albicans Candida* (NCAC) species such as *C. glabrata, C. tropiclis* and *C. parapsilosis* from other *Candida* species. Api Candida system and VITEK 2 system were used as a conventional diagnosis for the *C. glabrata, C. tropicalis* and *C. parapsilosis* than other *Candida* species.

### DNA extraction

Genomic DNA was extracted from the *C. glabrata, C. tropicalis* and *C. parapsilosis* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modifications. Briefly, 1 ml culture of *C. glabrata, C. tropicalis* and *C. parapsilosis* isolates grown for 20 hours at 37°C in YPD broth (Sigma, USA) were transferred to a 1.5 ml microcentrifuge tube. The samples were centrifuged at 13,000 rpm for 3 minutes to pellet the cells and the supernatant was removed, then the cells were resuspended thoroughly in 300 µl of 50 mM EDTA (Sigma, USA). 20 µl of 20 mg/ml proteinase K (Sigma, Missouri, USA) was added and gently pipet 4 times to mix, then the samples were incubated at 37°C for 30 minutes to digest the cell wall and cooled at room temperature. The samples were centrifuged at 13,000 rpm for 3 minutes, the supernatant was removed and 300 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added to the cell pellet, then gently pipet to mix. 100 µl of protein precipitation solution (wizard genomic DNA purification kit) was added and vortex vigorously at high speed for 20 seconds, then the samples were sit on ice for 5 minutes and centrifuged at 14,000 rpm for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5 ml microcentrifuge tube containing 800 µl of cold absolute ethanol and gently mixed by inversion until the thread-like strands of DNA form a visible mass, then centrifuged at 14,000 rpm for 10 minutes. The supernatant was carefully decanted and the tubes were drained on clean absorbent paper and 300 µl of room temperature 70% ethanol were added, then the tubes were gently inverted several times to wash the DNA pellet. The samples were centrifuged at 14,000 rpm for 2 minutes and all the ethanol was carefully aspirated. The tubes were drained on clean absorbent paper and the pellet was allowed to air-dry for 10 minutes, then 50 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added. 1.5 µl of RNase solution (wizard genomic DNA purification kit) was added to the purified DNA sample and the sample was vortex for 1 second, then centrifuged briefly in a microcentrifuge for 5 seconds to collect the liquid and incubated at 37°C for 15 minutes. The DNA was rehydrated by incubating at 65°C for 1 hour and the solution was periodically mixed by gently tapping the tube, then the DNA sample was stored at -20°C until use.

### DNA quantification

The extracted DNA from the *C. glabrata, C. tropicalis* and *C. parapsilosis* isolates were quantified spectrophotometrically at OD260/280 nm with ratios 1.3-1.6. The sensitivity of the (CGL1, CGL2), (CTR1, CTR2) and (CPA1, CPA2) primers were evaluated by PCR amplification for serial diluted concentrations (10ng-100ng) of purified genomic DNA isolated from *C. glabrata, C. tropicalis* and *C. parapsilosis,* respectively.

**Table 1. Name, sequence and the expected product size of *C. glabrata, C. tropicalis* and *C. parapsilosis* primers**

| *Candida* species | Name of primer | Sequence of primer (5'→3') | Expected product size (bp) |
|---|---|---|---|
| *Candida Glabrata* | CGL1 | TTATCACACGACTCGA CACT | ~423 |
| | CGL2 | CCCACATACTGATATGGCCTACA | |
| *Candida tropicalis* | CTR1 | CAATCCTACCGCCAGAGGTTA T | ~357 |
| | CTR2 | TGGCCACTAGCAAAATAAGCGT | |
| *Candida parapsilosis* | CPA1 | TTGGTAGGCCTTCTATATGGG | ~300 |
| | CPA2 | CCTATCCATTAGTTTATACTCCGC | |

### Multiplex PCR primers selection

The species-specific primers for ITS region of rRNA gene of *C. glabrata, C. tropicalis* and *C. parapsilosis* as the target gene for this study were selected according to (Guizhen and Thomas, 2002). These sets of unique primers were designed based on the conserved regions in *C. glabrata, C. tropicalis* and *C. parapsilosis*, primers were synthesized by Alpha DNA, Kanda. The name, sequence and expected product size of these species-specific primers for ITS region of rRNA gene are shown in (Table 1).

### Multiplex PCR Master Mix

The multiplex PCR reaction for detection of ITS region of rRNA gene for *C. glabrata, C. tropicalis* and *C. parapsilosis* was performed in 50 µl volumes containing 12 µl of nuclease free water, 20 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 1 µl of 20 pmol CGL1, CGL2 primers, 1 µl of 20 pmol CTR1, CTR2 primers, 1 µl of 20 pmol CPA1, CPA2 primers, and 4 µl of genomic DNA sample of each *C. glabrata, C. tropicalis* and *C. parapsilosis*. The mixes were overlaid with 2 drops of mineral oil.

### Multiplex PCR program

Multiplex PCR was carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by (Guizhen and Thomas, 2002). Briefly, the Amplification of ITS region of rRNA gene for *C. glabrata, C. tropicalis* and *C. parapsilosis* was carried out with initial denaturation at 96°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for (CGL1, CGL2), (CTR1, CTR2) and (CPA1, CPA2) primers for 30 seconds, and extension at 72°C for 30 seconds. The thermal cycles were terminated by a final extension for 15 minutes at 72°C.

### Multiplex PCR products analysis

The analysis of multiplex PCR product of ITS region of rRNA gene for *C. glabrata, C. tropicalis* and *C. parapsilosis* was performed on 1% agarose gels. The 100 bp DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The PCR product was stained with ethidium bromide and visualized by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

## DISCUSSION

### Isolation of *C. glabrata*, *C. tropicalis* and *C. parapsilosis*

In this study, the result of *Candida* species isolation showed that the first most common isolated species was *C. glabrata*, the second isolated species was *C. tropicalis*, the third isolated species was *C. parapsilosis* and the other samples were for other *Candida* species that isolated from female patients clinically diagnosed with vulvovaginitis in this study. The result of this study in agreement with results of different worldwide studies which conducted on *Candida* species that referred the first and second most common isolated species were *C. albicans* and *C. glabrata* (Abu-Elteen, 2001; Novikova *et al*., 2002; Okungbowa *et al*., 2003 Paulitsch *et al*., 2006; Grigoriou *et al*., 2006) and the prevalence of other *Candida* species had the same pattern in this study as seen in findings of the other studies (Fan *et al*., 2008, Ahmad and Khan, 2009; Odds, 1988).

### Culture on Sabouraud′s dextrose agar

The morphological characteristics of *C. albicans, C. glabrata, C. tropicalis* and *C. parapsilosis* isolates on plates of the Sabouraud′s dextrose agar containing chloramphenicol (50µg/ml) were matching with the morphological characteristics of *Candida* obtained by (Larone, 2002), they referred that the colonies of *Candida*, on the routinely used Sabouraud′s dextrose agar (SDA), are cream to yellow in colour, and depending on the *Candida* species, colony texture may be smooth, glistening or dry, or wrinkled and dull.

The result was in agreement with the findings obtained by different studies that showed the fungal cultures were carried out by conventional methods on routine media (such as Sabouraud′s dextrose agar). Any growth obtained was further identified by its temperature requirement, rate of growth, colony morphology and lactophenol cotton blue mounts (Glenn *et al*., 2005 and Koneman *et al*., 2006). Other study exhibit that the yeast identification was done on the basis of Indian ink preparation, germ tube production, sporulation on corn meal agar, urease production, sugar fermentation and assimilation and colour production on CHROM agar (Agarwal *et al*., 2011). Furthermore, the results of current study were consistent with the findings of (Prakash *et al*., 2013) that all the culture growths of the *Candida* species, *Trychophyton mentagrophytes* and *Trychophyton rubrum* which isolated from fingernail onychomycosis were identified on the basis of the culture characteristics on Sabouraud's Dextrose Agar (SDA) with antibiotics (chloramphenicol and cycloheximide), Lactophenol Cotton Blue (LCB) test, germ tube test, Dalmau method by using corn meal agar, sugar assimilation test and urease test.

**Culture on CHROM agar**

In current study, the observed phenotypic characteristic which refer to the colour of growing colonies of *C. albicans, C. glabrata, C. tropicalis* and *C. parapsilosis* isolates on the CHROMagar was similar to colours of colonies of these *Candida* species on the same medium that characterized by (Calderone, 2002), which were blue-green colonies for *C. albicans,* Pink-purple colonies for *C. glabrata,* dark blue colonies for *C. tropicalis* and white colonies for *C. parapsilosis*. In other study conducted by (Kathrin, 2000), to find out easy-to-perform selective isolation procedures to differentiate between closely related species *C. albicans* and *Candida dubliniensis*, oral rinses of human immunodeficiency virus (HIV)-infected patients were cultured on CHROMagar Candida, green colonies described as being indicative of *C. albicans* and dark green colonies described as being indicative of *C. dubliniensis*. Furthermore, the results of phenotypic characteristic of these *Candida* species on CHROMagar were consistent with the findings of (Odds and Bernaerts, 1994; Hospenthal, 2006) which showed that the CHROMagar medium can be used to reliably differentiate *C. albicans, C. tropicalis* and *C. krusei,* with a discriminatory power. Also the result of current study was matching with the finding of study conducted by (Odds and Bernaerts, 1994) reported that with CHROMagar medium, there was no delay in yeast detection and no change in the size of colonies compared with that obtained on SDA when the same clinical samples were plated onto the two culture media.

**VITEK 2 system**

Because relatively some of the phenotypic identification procedures are based on colorimetric or pH-based changes and usually require 18 to 24 h to identify organisms and some of them are based on changes in preformed enzymes, shortening the time necessary to make identification, the Bact/Alert instrument is designed to decrease the turnaround time for the identification of microoraganisms and use more conventional processes remains (O'Hara, and Miller, 2003). To find out appropriate diagnosis method for differentiation between *Candida auris* and *C. haemulonii* such as Vitek automated readings, different studies highlights that *Candida auris* remains an unnoticed pathogen in routine microbiology laboratories in India, as 90% of the isolates characterized by commercial identification systems misidentify this yeast as *C. haemulonii*. In the past 5 years, *Candida auris* has emerged as a significant pathogen in tertiary care general hospitals and a pediatric center in north and south India, representing 8.6% to 30% of cases of candidemia (Chowdhary *et al*., 2013 and Chowdhary, 2014). Also, accurate identification of the cryptic species *C. auris* is important in assessing the epidemiology and pathogenicity of the disease caused by this underreported pathogen in different geographic areas. In the past 5 years, *C. auris* fungemia has been reported from South Africa, South Korea, Japan, and India (Lee, 2011). All of the reports from these countries confer the major issue of notable elevated MICs for azoles and caspofungin in *C. auris* and its misidentification by phenotypic methods (Magobo *et al*., 2014). In fact, the erroneously elevated MICs by the Vitek 2 automated reading method not only may lead to inappropriate selection of antifungal therapy but also depict false rates of high antifungal resistance in epidemiological studies (Pfaller *et al*., 2007). Furthermore the study conducted by (Nageswari *et al*., 2013) showed that all the germ tube negative *Candida* spp. were speciated by the automated system (Biomerieux, Vitek 2 C) using YST identification, as well as AST, determined by YST-AST-01 card.

**API Candida system**

Different independent studies referred that the increasing incidence of yeast infections has stimulated the development of both manual and automated commercial systems for the identification of these organisms. The API 20C yeast identification system (BioMerieux, France) was one of the first commercial systems to be introduced for the purpose of yeast identification (Buesching *et al*., 1979 abd Land *et al*., 1979) and is now considered a reliable, proven system with which others are to be compared (El-Zaatari *et al*., 1990; Fenn *et al*., 1994; Davey *et al*., 1995; Espinel-Ingroff *et al*., 1998; and Heelan, 1998). However, even though it is faster than classical assimilation and fermentation methods, the API 20C system is still time-consuming to set up and read, requires up to 72 h of incubation, and gives results that are often difficult to interpret (Colin *et al*., 1999).

The results of current study matching with findings of other studies which exhibited that unlike the API 20C yeast identification system, the AUXACOLOR (Sanofi Diagnostics Pasteur, France) and API Candida systems (BioMerieux) are intended to identify only a limited range of taxa, comprising the most commonly encountered clinical pathogens. The AUXACOLOR system, it is claimed, is capable of identifying 26 species, while the API Candida system can identify 15 species,. Both systems have been reported to provide a simple, rapid, and accurate means of identifying clinical yeast isolates (Fricker-Hidalgo *et al*., 1996 and Hantschke, 1996). Also the result of this study goes together with results of study conducted by (Bineshian, 2015) to identify different *Candida* species (*C. albicans, C. glabrata* and *C. parapsilosis*) attributed to systemic candidiasis by cornmeal agar supplemented with tween-80, germ tube formation in serum, the API 20 C AUX yeast identification system, and polymerase chain reaction using species-specific primers for the MP65 65 kDa gene.

**DNA extraction**

The result of current study showed the important use of commercial kits such as genomic DNA extraction kit as a rapid extraction method for the genomic DNA comparing with conventional DNA extraction methods such as alkaline lysis, boiling and salting out methods, and this finding agree with finding of (Mahnaz *et al*., 2012) that they referred to interest use of the genomic DNA extraction kit (AccuPrep Bioneer Corporation) as an effective and rapid method for extracting the DNA. This eliminated the use of phenol-chloroform which is a cumbersome and tedious step of other PCR methods, resulting in significant improvements in the processing speed.

**Detection of ITS1 region of rRNA gene for *C. glabrata*, *C. tropicalis* and *C. parapsilosis* isolates by multiplex PCR**

*Candida* species are still the most common yeast infections worldwide. Hence, the reliable and rapid identification method of these species is a fundamental goal of microbiology laboratories. The multiplex PCR method is a highly sensitive and specific technique. Despite their demonstrated reliability, molecular methods have not been routinely used to identify *Candida* species (Tarini *et al*., 2010). The result of this study agrees with the study that performed by (Liguori *et al*., 2010) which compared different chromogenic and biological methods to PCR for *Candida* identification. They pointed out high incubation time, lack of experienced personnel, lower sensitivity and specificity, and lower discrimination power as disadvantages of other methods and suggested using them for screening and preliminary assays, while introduced the multiplex PCR as a precise and simple to implement method with no requirement of toxic and expensive chemical reagents. This cross-sectional study used a multiplex PCR method previously used by (Chang *et al*., 2001) to identify the *Candida* species in a sample of Iranian population. It has been previously shown that 30.7% of isolated *C. glabrata* are resistant to common antifungal therapies compared to 0.6% of *C. albicans*. Hence, including methods which can identify the non-*albicans* species is important and useful in choosing the appropriate treatment (Hazen *et al*., 2003 and Tortorano *et al*., 2004). The other advantage of multiplex PCR method in this study agree with study performed by (Mahnaz *et al*., 2012) which demonstrated that another advantage of multiplex PCR method is its ability to identify more than one species in a single specimen. This advantage also agree with the study performed by (Markoulatos *et al*., 2002) which demonstrated that multiplex PCR requires that primers lead to amplification of unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of reaction conditions.

**Conclusion**

This study has demonstrated the efficacy of the ITS1 region of rRNA gene to detect the *C. glabrata, C. tropicalis* and *C. parapsilosis* (Non-*Candida albicans Candida* species) in the clinical samples. This multiplex PCR-based test is a sensitive, rapid, and a applicable method to detect cadidial etiology in the female patients clinically diagnosed with vulvovaginitis when compared with conventional laboratory diagnosis of these Non-*Candida albicans Candida* species.

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