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Research Article

Identification of *Candida* Species Associated with Vulvovaginal Candidiasis by Multiplex PCR

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Background. Vulvovaginal candidiasis is a common infection. The aim of this study was to identify the species of vaginal *Candida* isolates by using multiplex PCR technique. *Methods*. 191 isolates from patients admitted to Mahdieh hospital were identified. The vaginal swab specimens were cultured on Sabouraud Dextrose Agar. The ITS1 region between the 18S and 5.8S rRNA genes and a specific DNA fragment within the ITS2 region were amplified. The multiplex PCR products were separated by electrophoresis in 2% agarose gel, visualized by staining with ethidium bromide, and photographed. Descriptive statistics, Chi-square test, and Spearman correlation were used to summarize the findings. *Results*. *C. albicans* and *C. glabrata* were the most common species isolated from the specimens. A mix of *C. glabrata* and *C. albicans* was the most common mixed infection isolated from the samples. The analysis revealed a significant positive association between older age and infection with *C. glabrata* isolates (Spearman's rho $= 0.89$, $P = 0.015$). *Conclusion*. Multiplex PCR is a fast, yet reliable method to identify *Candida* species. *C. albicans* and then *C. glabrata* are the two most common causes of vulvovaginal candidiasis. The number of mixed fungal infections is higher among Iranian population compared to international reports.

1. Introduction

Candida species are the second most common cause of vulvovaginitis worldwide [1]. The prevalence of vulvovaginal candidiasis (VVC) is increasing due to the extensive utilization of broad-spectrum antibiotics as well as increased cases of immunocompromised patients [2, 3]. Nearly 75% of women over 25 years of age, reported to have at least one episode of physician approved VVC during their lifetime and 5% experienced recurrent type; which is defined by getting infected for at least 4 times in a one-year period [4]. However, 20–50% of women have *Candida* species in their vaginal flora without showing any clinical symptoms [4, 5]. *C. albicans* is the most common and clinically relevant species that accounts for 85–90% of VVC [4]. However, there has been a significant trend towards the emergence of other species such as *C. glabrata*, *C. krusei,* and *C. parapsilosis* which ironically show more resistance to the first line antifungal treatments [6]. Hence, the differentiation of diverse species of *Candida* in the laboratories seems necessary. Traditionally, the identification and classification of *Candida* species were done by time consuming and unreliable methods such as serotyping [7], colony morphotyping [8], conventional culture techniques, and morphological and biochemical analysis [9]. Nonetheless, the improvements in molecular assay technology for identifying *Candida* species, such as randomly amplified polymorphic DNA analysis (RAPD), has overcome these limitations during the last couple of years. However, methods such as single and direct PCR or multiplex PCR have not been used extensively despite being highly sensitive

and specific with a shorter turn-around time [10–13]. Multiplex PCR is a rapid diagnostic assay which combines many specific species primers in one PCR tube. Hence, it could be used to identify more than one species in a specimen simultaneously [14]. Very little is known regarding the epidemiology of VVC in Iran. The aim of this study was to identify different species of Candida in an Iranian patient population by using different methods. Multiplex PCR method was also evaluated as a rapid and reliable method to identify *Candida* species by comparing the results with the traditional methods such as germ tube formation in serum, chlamydospore production on Corn Meal Agar (CMA), and carbohydrate absorption.

2. Materials and Methods

Participants of this study were women with signs and symptoms of VVC, who were admitted to the gynecology clinic in Mahdieh Educational Hospital in Tehran, Iran during a 2-year period from March 2006 to 2008. Participants who experienced the signs of VVC less than four times (including their latest admission to the clinic) in the previous year were categorized in nonrecurrent VVC (NRVVC), and those who experienced the vaginal itch and secretions equal or more than four times in the previous year were considered recurrent VVC (RVVC) after being confirmed by finding three positive cultures of VVC within a year in their medical records. Specimens were collected from the first 100 consecutive patients with NRVVC and likewise for the first 100 consecutive patients with RVVC. Each patient was tested only once at her first visit to the clinic during the two-year period of the study. Out of a total 200 collected specimens, 25 were excluded from the study due to contamination or having no signs of fungal growth. Hence, the final number of specimens was 175. To further evaluate the risk factors for VVC, we also asked our participants to complete a questionnaire regarding their demographic and behavioral characteristics. This study was approved by Iran's National Research Ethics Committee. All of the patients signed a written consent form before participating in the study and patients' confidentiality was strictly protected. Furthermore, no complication was seen after taking the vaginal samples.

Vaginal sampling of the participants performed by using a sterile swab by the principle researcher and was cultured simultaneously onto sabouraud dextrose agar medium. *Candida* species colonies were also identified by germ tube formation in serum, chlamydospore production on CMA, and carbohydrate absorption using the API 20 C-AUX kit (bioM'erieux, Paris, France), results of which have already been published elsewhere [15]. Colonies were placed in transport medium at room temperature before being processed for PCR analysis.

DNA extraction and purification were performed using a Genomic DNA Extraction kit (AccuPrep Bioneer Corporation) based on the guidelines. Multiplex PCR was performed using the PCR premix kit (AccuPower Bioneer Corporation) with a total reaction volume of a 50 μL consists of 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM deoxyribonucleoside triphosphates (0.2 mM each), 4 μM primers (0.16 μM each), and Taq DNA polymerase (1 U). The primers used in this reaction were synthesized at TAGC (Berlin, Germany) including universal primers (ITS1 [5′-TCCGTAGGTGAACCTGCGG-3′] and ITS2 [5′-GCTGCGTTCTTCATCGATGC-3′]) [12] and *C. albicans*-specific primers (CA3 [5′-GGTTTGCTTGAAAGACGG-TAG-3′] and CA4 [5′-AGTTTGAAGATATACGTGGTAG-3′]). A conserved portion of the 18S rDNA region, the adjacent ITS1, and a portion of the 28S rDNA region were amplified, using the ITS1 and ITS2 primers. A portion of the ITS2 region of *C. albicans* was amplified by including CA3 and CA4 in the PCR mixture. PCR amplification process was carried out with an Eppendorf thermal cycler under the following conditions: initial denaturation (94°C, 3 min); 35 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min); final extension (72°C, 5 min). PCR products were analyzed by electrophoresis through a 2% agarose gel (Roche) containing ethidium bromide (Sigma), and UV visualization were performed according to the protocols provided (UVdoc, GAS9000, England). The length of the bands was measured by UVIsoft software. Positive controls were included in each PCR experiment and consisted of one strain of each *C. albicans* ATCC14053 (218 or 219, and 110 bp), *C. glabrata* CBS2175 (482 or 483 bp), *C. parapsilosis* CBS2195 (229 bp), *C. tropicalis* CBS94 (218 bp), and *C. krusei* CBS573 (182 bp). Pyrogen-free water was used as negative control. Two DNA bands were identified for *C. albicans* while one DNA band was corresponded to other *Candida* species. An assessment of the different species of RVVC and NRVVC was also made. Descriptive statistics, chi square test, and Spearman's correlation were used to analyze the data.

3. Results

The mean age (±SD) of the 175 participants was 32.4 (±8.2) years. There was no significant difference between the mean age of women with RVVC and those with NRVVC ($P < 0.9$). There was a significant association between the age of participants and detection rates of *C. albicans* ($P < 0.05$). The results of the analysis indicate that older participants were less likely to be infected with *C. albicans*. However, as depicted in Figure 1, they were most prone to get infected with *C. glabrata*.

Figure 2 shows the results of the multiplex PCR method.

Among vaginal samples, 89.7% contained only one species of *Candida* and 10.3% contained more than one species of *Candida*. The prevalence of different species of *Candida* was as follow: *C. albicans* (65.1%), *C. glabrata* (13.1%), *C. tropicalis* (6.2%), *C. krusei* (4%), *C. guilliermondii* (0.6%), *C. parapsilosis* (0.6%), mixed infection of *C. glabrata* and *C. albicans* (5.7%), *C. parapsilosis* and *C. albicans* (1.1%), *C. krusei* and *C. albicans* (0.6%), *C. albicans*and *C. tropicalis* (0.6%), *C. glabrata* and *C. tropicalis* (0.6%), *C. krusei* and *C. tropicalis* (0.6%), *C. krusei* and *C. glabrata* (0.6%), and a combination of *C. glabrata*, *C. krusei*, and *C. albicans* (0.6%). Only one species of *Candida* was identified in 90.2% of participants with NRVVC while 9.8% of them were infected with more than one species. Also,

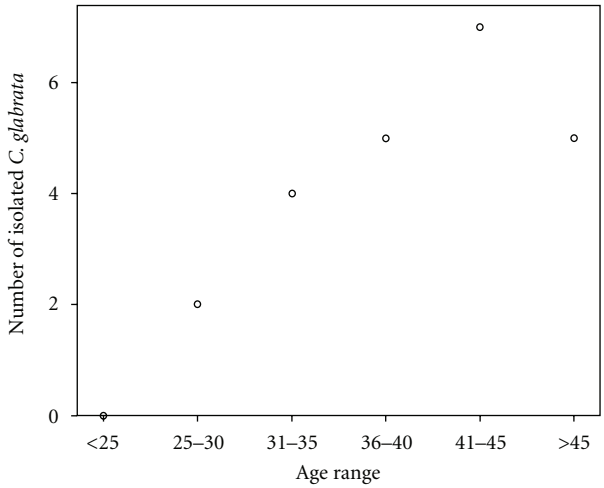

Figure 1: Correlation of participants' age range and isolated *C. glabrata*.

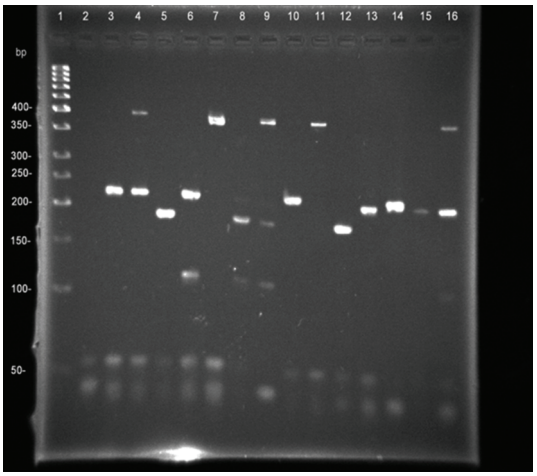

Figure 2: Multiplex PCR using primers ITS1, ITS2, CA3, and CA4. Lane 1: 50-bp DNA ladder. Lane 2: negative control. Lanes 3 to 8, 11 to 13 and 15 to 16: eleven clinical isolates. Lane 9: mixed reference strains *C. krusei* CBS573, *C. albicans* ATCC14053, and *C. glabrata* CBS2175, Lane 10: reference strain *C. tropicalis* CBS94, and Lane 14: reference strain *C. parapsilosis* CBS2195.

89.7% of those with RVVC were infected with one species compared to 10.8% who were infected with more. There was no statistically significant difference between these groups ($P < 0.3$). Chi-square test showed no significant difference between the prevalence of *Candida* species among recurrent and nonrecurrent groups ($P < 0.5$). A more detailed comparison of demographical and behavioral characteristics of the participants has already been published elsewhere [15]. The results of the multiplex PCR method perfectly matched the results of the germ tube test and chlamydospore production on CMA and API 20C-AUX kit.

4. Discussion

C. albicans is still the most common yeast infection worldwide. Hence, the reliable and rapid identification method of this species is a fundamental goal of microbiology laboratories. The multiplex PCR method is a highly sensitive and specific technique based on the results of the previous studies [16]. Despite their demonstrated reliability, molecular methods have not been routinely used to identify *Candida* species. Liguori et al. compared different chromogenic and biological methods to PCR for *C. albicans* 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 [17]. This cross-sectional study used a multiplex PCR method previously used by Chang et al. [12] 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* [6, 18]. Hence, including methods which can identify the nonalbicans species is important and useful in choosing the appropriate treatment. The results of this study showed that *C. glabrata* is more common in women at older age while other species are more common among the young population. This finding is in agreement with previous studies [19–22] and could be due to the development of antifungal resistance, the immune response, or hormonal changes among women of older age [19]. However, the result could be due to an unanticipated bias, and further studies seem essential to solely address this observation. The results of this study showed that 10.3% of participants were infected with more than one species of *Candida* which is a higher rate compared to the other studies conducted in China [23], United States [24], and Jordan [25]. The first and second most common isolated species in this study were *C. albicans* and *C. glabrata*which was in agreement with similar studies conducted worldwide [23, 25–31]. The prevalence of other species had the same pattern in our study as seen in earlier findings [25, 32–37]. Finally, of interest is using the Genomic DNA Extraction kit (AccuPrep Bioneer Corporation) as an effective and rapid method for extracting the DNA in 35 minutes. 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 (the whole process could be completed in less than 6 hours). Another advantage of Multiplex PCR method is its ability to identify more than one species in a single specimen. Despite its strengths, our study was subject to some limitations such as low number of participants due to the lack of budget and personnel and collecting samples from patients admitted to one hospital which can affect the results of this study. Therefore, the results of our study should be used with caution and further research is recommended.

5. Conclusion

The results of this study showed that *C. albicans* is the most common *Candida* species in VVC among women followed

by *C. glabrata* which has a higher prevalence among older women. A multiplex PCR method was used to identify the *Candida* species which seemed to be a reliable, rapid, and cost effective technique since it only requires PCR components and a commercial DNA extraction kit.

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