Identification of *Candida* species isolated from vulvovaginal candidiasis using PCR-RFLP

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ABSTRACT

Vulvovaginal candidiasis (VVC) is a common disease among women worldwide, therefore, accurate and rapid diagnosis of causative agents based on molecular techniques utilizing amplification of target DNA is highly recomendad for epidemiological purposes and for effective treatment. The aim of this study was to identify clinically *Candida* species from VVC patients by restriction fragment length polymorphism (PCR-RFLP). A total of 155 patients with suspicious symptoms of VVC were screened. *Candida* strains isolated from specimens initially were identified by phenotypic methods and confirmed by molecular approaches based on PCR-RFLP. Fifty one (34%) strains of *Candida* were obtained from specimens collected from VVC patients. *C. albicans* was the most frequently isolated species (86.2%) followed by non-albicans, including *C. glabrata* (7.9%), *C. kefir* (3.9%), and *C. tropicalis* (1.9%). The restriction patterns of each *Candida* species were perfectly specific. The identification of *Candida* species in VVC due to developing antifungal resistance is very significant for appropriate treatment and to prevent the spread of VVC.

1. Introduction

Vulvovaginal candidiasis (VVC) is a syndrome characterized by vulvovaginal inflammation due to *Candida* infection (Geiger et al., 1995). The actual incidence of VVC remains unknown. Estimates suggest that 75% of women will have at least one episode of VVC during their life span and 50% of them experience multiple episodes as well (Sobel, 1993). The incidence of VVC increased in risk groups with uncontrolled diabetes, immunocompromised hosts, patients using broad spectrum antibiotics and corticosteroids or patients with severe underlying diseases (Sobel, 2002, Sobel, 1993). Roughly 80% to 95% of VVC are caused by *C. albicans* which follows by non-albicans *Candida* species including *C. glabrata* and *C. tropicalis* (Ferrer, 2000, Sobel et al., 2001). In addition, the widespread use of antifungal agents has caused the pathogens in VVC to change, and recurrent vulvovaginal candidiasis (RVVC) has been imputed to non-albicans species (Sobel, 1985). Hence, the identification and discrimination of ethological agents for early treatment, and preventing the invasion is highly recommended. Due to the high degree of phenotypic similarity

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between the most of *Candida* species, identification problems are imminent (Bohannon, 1998). The use of traditional methods for identification and typing of clinical *Candida* isolates are time consuming and dependent on phenotypic expression, that makes them potentially unreliable (Wilson et al., 2001). Alternative molecular assay with high specificity, reproducibility and sensitivity are necessary (Mirhendi et al., 2006, Mirhendi et al., 2001). Thus the objective of this study was to identify clinically *Candida* species from VVC patients by restriction fragment length polymorphism (PCR-RFLP).

2. Materials and Methods

2.1. Yeast isolates

One hundred and fifty five middle age women with the complaints of pruritus, pain and vaginal discharge were analyzed for vulvovaginal candidiasis. Two sterile cotton tipped swabs were used to collect specimens from the lateral wall of vagina of each woman. One of them was used to direct wet-mount microscopy using 10 percent potassium hydroxide solution. The other was used for culture onto sabouraud dextrose agar (SDA, Difco) with chloramphenicol (SC), and incubated at 37°C for 2 days in dark. Yeast growth was semi quantitatively noted as none, + (<10 colonies), ++ (10–50 colonies), or +++ (>50 colonies) (Solimani et al., 2014). These strains were initially identified by phenotypic and physiologic criteria including colony color on CHROMagar *Candida* medium (CHROMagar Company, Paris, France), germ-tube tests in serum at 37ºC for 2–3 hours, microscopic morphology on Corn-meal agar (CMA, Difco) with 1% tween 80. One colony on each identification strains was sub-cultured for molecular investigation.

2.2. DNA Extraction

Genomic DNA was extracted using the method of glass bead disruption (Mirhendi et al., 2001). Briefly, 300 μl of lysis buffer (10 mM Tris, 1 mM EDTA (pH 8), 1% SDS, 100 mM NaCl, 2% triton X-100), 300 μL of phenol-chloroform (1:1) solution and equal to 300 μl of 0.5 mm diameter glass beads, were added to yeast. After 5 min of vigorous shaking which followed by 5 min centrifugation at 10000 rpm, the supernatant was isolated and transferred to a new tube and equal volume of chloroform was added, mixed gently, centrifuged and its supernatant was transferred to a new tube. For alcohol precipitation, 0.1 ml volume sodium acetate (pH 5.2) and 2.5 ml volume of cold absolute ethanol were added and the mixture was gently shaken and centrifuged at 10000 rpm for 10 min at 4°C. After washing with 70% ethanol, the pellets resuspended in 100 μl TE buffer (10 mM Tris, 1 mM EDTA) and were stored at -20°C prior to use.

2.3. RCR- RFLP and sequencing analysis

The PCR-RFLP method was performed as previously described (Mirhendi et al., 2006). For PCR amplification and sequencing, fungal-specific universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to amplify the ITS regions of the rDNA. A PCR was performed for each 50-μL isolate using 5 μL of 2 mM 10× PCR buffer, 5 μL of 2 mM dNTPs (BIORON GmbH, Germany), 3 μL of 25 mM MgCl2, 1 μl of each primer at 25 pmol,, 0.4 μL of 5 U/μL Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 2 μL of template DNA and 32.6 μL of doubled distilled water. Each component was added to a 200-μL PCR tube and mixed thoroughly. The PCR reaction was conducted at 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min. In the first cycle, the denaturation step was 94°C for 5 min and in the final cycle the final extension step was 72°C for 7 min. PCR products were digested in a final reaction volume of 15μl containing 3 μl water, 1.5μl buffer, 1U of restriction enzyme *Msp I* and 10 μl PCR product at 37˚C for 2h. Amplified and digested products were visualized by 1.5% and 2% agarose gel electrophoresis in TBE buffer (0.09 M Tris, 0.09 M boric acid and 2 mM EDTA, pH 8.3), respectively, and stained with ethidium bromide (0.5 μg/ml) and photographed. The size of DNA fragments determined directly with comparison of

molecular size marker and distinct banding patterns which demonstrated in similar studies. Sequence data were aligned using MEGA 5.05 (http://www.megasoftware.net/) and Bioedit ver. 7.0.9 (http://www. mbio. ncsu. Edu /BioEdit /bioedit. html) (Alignment, BioEdit Sequence 2011) software packages and compared with GenBank database using the BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

Of 155 patients with suspicious symptoms of VVC, culture for *Candida* species was positive in 51 (34%) patients. After the digestion with *MspI* enzyme the results were evaluated based on the sizes of PCR products for *Candida* species, i.e., 297, 238 bp for *C. albicans*; 557,

314 bp for *C. glabrata*; 340, 184 bp for *C. tropicalis*; 722 bp for *C. kefir.* The ITS rDNA regions of all tested isolates were successfully amplified. The digestion of ITS region of *Candida* species by *MspI* enzyme created bands for *C. albicans*, *C. glabrata*, *C. tropicalis,* and *C. kefir.* Figure 1 demonstrates the patterns of PCR-RFLP for *Candida* strains after digestion with *Msp*I. As shown, the fragment lengths were exactly the same as the estimated sizes in the computational sequence analysis. Results show that *C. albicans* was the most frequently isolated species 44 (86.2%) followed by non-*albicans* included *C. glabrata* 4(7.9%), *C. kefir* 2(3.9%), and *C. tropicalis* 1(1.9%). The restriction patterns of each *Candida* species were perfectly specific.
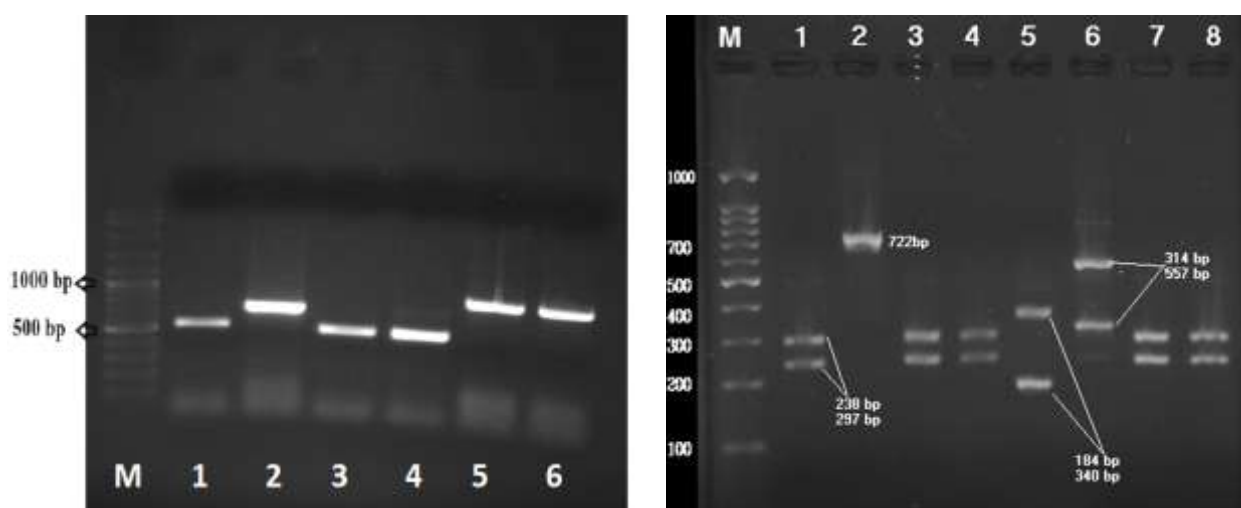

Figure 1. PCR products of ITS1/ITS4 amplification in *Candida* spp (Left). Patterns of PCR products of *Candida* isolates after digestion by the restriction enzyme *MspI* (Right). Lanes of 1, 3,4,7,8 represent *C. albicans*; 2, *C. kefir*; 5, *C. tropicalis*; and 6, *C. glabrata*. Lane M is 100 bp ladder molecular size markers.

4. Discussion

The identification of clinical important *Candida* strains is of priority to better treatment and early eradication (Corsello et al., 2003). In the present study, 51 (34%) strains of *Candida* were obtained from specimens collected from VVC patient. This result is in agreement with the study conducted by Bauters et al., who isolated *Candida* species in 20.1% (Bauters et al., 2002). In other study, reported 18.5%

prevalence of VVC in a community setting (Mohanty et al., 2007). Our finding revealed that *C. albicans* as predominant species, followed by *C. glabrata* (7.9%), *C. kefir* (3.9%), and *C. tropicalis* (1.9%) isolated from VVC patients. Prior studies in Iran and other countries revealed *C. albicans* to be the most common species in VVC patients (76 to 89%), followed by *C. glabrata* (Corsello et al., 2003, Regulez et al., 1994, Rad et al., 2011, Mahmoudi Rad et al., 2012). In the past years, several studies have

reported an increasing trend in the occurrence of non-*C. albicans* species over the time (Chaim, 1997, Geiger et al., 1995, Sobel et al., 1998). Guzel et al. reported a 50% non-*albicans Candida* species prevalence in their mixed acute and chronic vaginitis patient population (Guzel et al., 2011). Consistent with the results of previous studies, *C. glabrata* is the most commonly reported as non *C. albicans* species in this population (Bankar et al., 2012, Cetin et al., 2007, Fidel et al., 1999). Prior studies in Iran showed that *C. glabrata* was the second most common species in the VVC patients (Mahmoudi Rad et al., 2012, Mahmoudi Rad et al., 2011, Mahmoudabadi et al., 2010). Diba et al. shoed that *C. glabrata* and *C. tropicalis* as the most common non-*Candida albicans* isolates, by using PCR-RFLP (Diba et al., 2012). It has been confirmed that efficacy of several antifungal drugs such as fluconazole against *C. glabrata* which used in clinical therapy is low (Fidel et al., 1999). Molecular approaches are crucially required for the identification of *Candida* species in order to treat the patients suffering from candidiasis (Roudbary et al., 2013). In this study, we applied a PCR-RFLP method for identification of *Candida* species in VVC patients and similar to other studies, showed this technique is rapid, sensitive, and reliable and might be also used in clinical laboratories to identify clinically important *Candida* spp.

5. Conclusion

In this study we found that in PCR-RFLP method, restriction patterns of each *Candida* species were perfectly specific. The molecular identification of *Candida* species in VVC due to developing antifungal resistance is very significant for appropriate treatment and to prevent the spread of VVC. We recommend further investigation into the most reliable and cost effective means of identification.

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Refereces

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