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Molecular identification and antifungal susceptibility of *Candida albicans* isolated from Vulvovaginitis Candidiasis

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ABSTRACT

Vulvovaginal candidiasis is caused by the increasing number of *Candida* species as normal flora in the vagina. To assess the transmission rout as well as to determine the suitable antifungal drugs for treatment, the exact identification of *Candida* species is crucial. Therefore, earlier detection of infection allows quick initiation of antifungal therapy with a greater probability for improved survival. The aim of this study was to evaluate the identification of *Candida albicans* using restriction fragment length polymorphism (RFLP) and to determine the *in vitro* susceptibility against four antifungal drugs. In this study, 100 clinical samples were obtained from the patients with suspected Vulvovaginal Candidiasis. Early identification of the grown yeasts was performed by physiological tests. Universal primers used to amplify the internal transcribe spacer region. Subsequent restriction enzyme analysis of PCR products was done using *Msp*1 and *Bln*1 which allows us to identify the most medically important *C.albicans* and *C.dubliniensis*. Antifungal susceptibility test was performed according to the CLSI M27-A3 broth microdilution method, and minimal inhibitory concentrations were determined for Nystatin, Fluconazole, Amphotericin B and Itraconazole. Fifty eight of 100 *Candida* species were isolated from suspected samples in which 36 isolates were identified as *Candida albicans*/ *C.dubliniensis* by using PCR-RFLP method as well as physiological test. Among them, only 1 isolate was identified as *C.dubliniensis*. All *C.albicans* isolates were susceptible to Amphotericin B and Nystatin. However, 5 *C.albicans* isolates were resistant to Fluconazole with MICs ≥ 64 µ g/ml (13.89%) and 4 isolates were resistant to Itraconazole with MICs ≥ 1 µ g/ml (11.11%). *C.albicans* is still the predominant species causing *Candida*-related infections. Nevertheless, the number of isolated yeasts from other species is growing gradually. Identification of *Candida* at species level is very important due to the increasing trend in the number of *Candida* isolates which have high MICs against antifungal agents.

1. Introduction

Vulvovaginal candidiasis is caused by the increasing number of *Candida* species as the normal flora in the vagina (Ferrer, 2000). Several predisposing factors such as antibiotic use or the use of oral contraceptives, pregnancy, personal habits, sexual activity, and uncontrolled diabetes make the women more susceptible to develop the infection (SOBEL, 1993). The symptoms such as thick white discharge with itching, congestion and redness of the skin friction in the vaginal area can be observed

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in patients (Sobel, 1997). There are several species of *Candida* causing Vulvovaginal Candidiasis, including *C.albicans, C.parapsilosis, C.tropicalis, C.glabrata, C.krusei, C.guilliermondii* and *C.dubliniensis*. Although the most common causative agent is *C.albicans*, it has been reported that recurrent vulvovaginitis is caused by non-*albicans Candida* species in 15-47% of patients (Ayati et al., 2012, Fidel and Sobel, 1996). The widespread use of antifungal drugs is considered as a reason for increasing the incidence of vulvovaginitis elucidated by non-*albicans* species.

The regimen of Imidazole antifungal may trigger imbalance in the vagina in a way that lead to an overgrowth of other *Candida* species. Most non-*albicans Candida* species are resistant or have high MICs against azole antifungal drugs, however, resistance in *C.albican*s isolates have been reported (Richter et al., 2005, Sanglard and Odds, 2002). To determine the suitable antifungal drugs, the exact identification of *Candida* species is crucial. Therefore, earlier detection of infection allows quick initiation of antifungal therapy with a greater probability for improved survival and reduced failure. PCR technology proposes potentially earlier detection, as well as species identification of fungal pathogens, allowing earlier initiation of antifungal therapy. PCR-RFLP is a PCR-based method which is able to distinguish *Candida* species with high level of sensitivity and specificity (Hossein et al., 2011, Mohammadi et al., 2011, Mirhendi et al., 2006, Mirhendi et al., 2001). In this study we used PCR-RFLP method for recognition of *C.albicans*, in addition to evaluate the antifungal susceptibility pattern against some antifungal drugs.

2. Matherials and Methods

2.1. Samples

From 2012 to 2013 (about 12 months), 100 vulvovaginitis suspicious clinical samples were collected from sexually active women referred to several hospitals Gonbad-Qabus, north-east of Iran. Patients suffered from erythema, edema, excoriations, fissures and white vaginal discharge. At the early stage of isolation, direct examination was performed by scrubbing vaginal swab samples with 15%KOH as well as culture on Sabouraoud's dextrose agar (HiMedia, India) supplied with both cyclohexamide and chloramphenicol. Cultures were then incubated at 30ºC for 48-72h. Totally, 58 grown yeast isolates were subjected to species identification. All grown yeasts were cultured on CHROMagar Candida (Himedia, India) as well and incubated at 35º C for 48 h.

2.2. Antifungal Susceptibility test:

The Susceptibility test of the antifungal agents (Nystatin, Amphotericin B, Fluconazole and Itraconazole) was conducted according to the Clinical and Laboratory Standards Institute M27-A3 document (Espinel-Ingroff et al., 2009). Amphotericin B and Itraconazole (Sigma Alderich, Company Germany) was prepared in Dimethyl Sulfoxide sterile (DMSO) whereas Fluconazole and Nystatin were dissolved in water. For the susceptibility test, RPMI 1640 (with glutamine, bicarbonate-free, and containing phenol red as the pH indicator) (Sigma) was used as a medium. The final concentrations were in the range 0.063–64 μg/ml for fluconazole, 0.016–16 μg/ml for Amphotericin B and Itraconazole (Espinel-Ingroff et al., 2009). Each *C.albicans* was studied twice for antifungal agents. Prior to testing, the isolates of *C.albicans* were grown on Sabouraud agar plates for 24 h at 35°C. The yeast suspensions were prepared in Normal saline after 24h of incubation, obtaining an initial concentration of 1 to 5×10^6 cell/ml (adjusted spectrophotometrically (Eppendorf) at 530 nm to match the turbidity of a 0.5 Mc Farland standard). These inoculums were diluted in RPMI 1640 medium, containing L-glutamine, and no sodium bicarbonate (Sigma Alderich, USA) morpholine-propanesulfonic acid was used as buffering agent (*Sigma-Aldrich*, Germany). The final cell density was 0.5×10^3 to 2.5×10^3 cell/ml. The cultured plates were incubated at 35°C for 48h. The MICs endpoints for Amphotericin B, Itraconazole were determined with the aid of a reading mirror as the lowest concentration of drug that inhibited recognizable growth (100% inhibition) and for fluconazole as the level which induced a prominent reduction of growth (≥ 50% inhibition compared to drug-free growth control). Two fungal strains were included in each assay for quality control *C.albicans* ATCC10231 used as susceptible strain and *C.albicans* ATCC76615 was used as resistant strain to antifungal agents.

2.3. DNA Extraction:

Total genomic DNA was extracted using the phenol-chloroform extraction method.

Briefly, 300 microlitre of lyses buffer [10 mM EDTA (pH 8), 1% SDS, 100 mM NaCl, 2% Triton X-100], 300 microlitre of phenol-chlorophorm (1:1) solution and 300 microlitres of 0.5 mm diameter glass beads, were added to fungal pellet. After 5 minutes of vigorous shaking and 5 minutes centrifugation in 10000 rpm, the supernatants were isolated and its DNA were precipitated by 0.1 volume sodium acetate (pH 5.2) and 2.5 volume cold absolute ethanol. After centrifugation for 10 minutes at 4°C and washing by 70% ethanol, the pellet resuspended in 50 microlitre TE buffer (10 mM tris, 1 mM EDTA pH 8) and stored at -20°C until using for PCR amplification (Holm et al., 1986).

2.4. PCR Amplification

The PCR assay was performed using 2 µL of the test sample (about 2 ng) in a final volume of 25 µL. The PCR mixture consisted of 10 mM Tris-HCl; master mix 1X; 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM each of dATP, dCTP, dGTP, and dTTP; 0.2 mM each of primers (ITS1: 3' – TCC GTA GGT GAA CCT GCG G - 5' and ITS4: 3' – TCC TCC GCT TAT TGA TAT GC- 5'); and 1 µL of Taq DNA polymerase (White et al., 1990). Thirty-five cycles of amplification were performed in an Eppendorf PCR (Eppendorf 5331 Mastercycler, Germany). The initial denaturation was performed at 94°C for 5 min; there after, each cycle consisted of a denaturation step at 94°C for 30 s, an annealing step at 56°C for 1 min, an extension step at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The amplified products were visualized on 1.5% Agarose gel run in Tris-borate-EDTA (TBE) buffer (0.09 M Tris, 0.09 M boric acid, and 20 mM EDTA; pH 8.3) and stained with 0.5 µg/mL Ethidium bromide (0.5µl). After the gels were stained with Ethidium bromide (0.5 µg/ml) and photographed by ultraviolet photography.

2.5. RFLP-analysis

The enzyme used in this study was *Msp*1 (Fermentase, USA). Digestion was performed by incubating 8.5 µl of PCR products that obtained by ITS1 and ITS4 primers with 0.5 µl of each enzymes at 10 U in a final reaction volume of 10 µl at 37ºC for 3 h. Restriction fragment were separated by 3% Agarose gel in TBE buffer for 1 h at 100 V. The gel was stained with Ethidium bromide (0.5 µg/ml) and photographed by ultraviolet. The size of DNA fragments determined directly with comparison of molecular size marker and distinct banding patterns which demonstrated in similar studies (Hossein et al., 2011; Mohammadi et al., 2011).

3. Result

Out of 100 suspicious vaginal samples, 58 yeast colonies were grown. Physiological tests such as germ tube and chlamydospore production as well as forming green color on CHROMagar medium were performed to distinguish *C.albicans*/ *C.dubliniensis* isolates from non-*albicans* one. Accordingly, among the grown colonies, 36 isolates were identified as *C.albicans*/ *C.dubliniensis*. *Candida albicans* ATCC10231 and C.*krusei* ATCC6258 was used as quality control.

3.1. PCR Amplification and RFLP-PCR analysis

All 36 strains identified as *C.albicans*/ *C.dubliniensis* physiologically were confirmed by use of PCR-RFLP method. The region of ITS was successfully amplified using primers ITS1 and ITS4, providing a single PCR product of approximately 535 bp (Fig-1).Using *Msp*I, *C.albicans* and *C.dubliniensis* produce the same banding pattern, two fragments of 240bp and 300bp; however, *Bln*I restriction enzyme can distinguish these two species successfully. Using *Bln*I, *C.dubliniensis* produce two fragments of about 200bp and 335bp; however, there are no cutting sites for *C.albicans*. Here all 35 *C.albicans* species were confirmed by PCR-RFLP and only one isolate of *C.dubliniensis* was isolated using *Bln*I.

3.2. Antifungal susceptibility test

Results are presented in terms of MIC inhibiting 50% of isolates (MIC50), MIC inhibiting 90% of isolates (MIC90) and MIC range values (minimum-maximum) for all *C.albicans* and antifungal drugs. The MICs of all *C.albicans* isolates (n = 35) against

Amphotericin B and Nystatin were low, ranging between 0.016–4 μg/ml and 1–4 μg/ml, respectively. We found high MICs range for fluconazole and Itraconazole (range 2–64 μg/ml and 0.125–4 μg/ml, respectively). Only 5 (13.88%) isolates showed MIC≥ 64 and were resistant to Fluconazole. Moreover, four (11.11%) isolates were resistant to Itraconazole with MIC ≥ 1 μg/ml. Table 1, summarizes the results of in vitro antifungal susceptibility of four antifungal drugs against isolates of *C.albicans*.

Table 1. The results of in vitro antifungal susceptibility of four antifungal drugs against isolates of *C.albicans*.

Antifungal	frequency			MIC **μg/mL**	
	susceptible	resistant	MIC range	MIC50	MIC90
Amphotericin B	35	0	0.016– 4	0.25	0.25
Nystatin	35	0	1– 4	2	4
Itraconazole	31	4	0.016-16	0.25	0.5
fluconazole	30	5	0.5-64	1	1

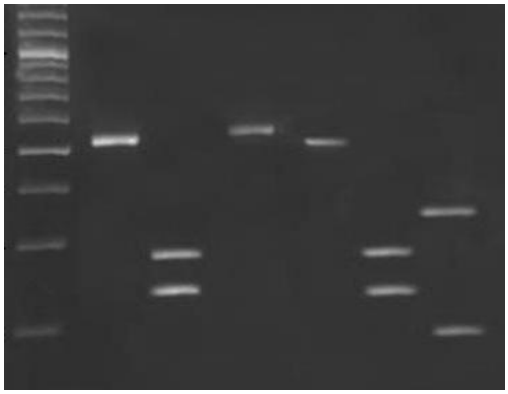

Figure 1. PCR products and *Candida* spp banding patterns with enzymes MspI, BlnI: 1) *C.albicans* PCR product, 2) *C.albicans* PCR product after digestion with MspI, 3) *C.albicans* PCR product after digestion with BlnI, 4) *C.dubliniensis* PCR product, 5) *C.dubliniensis* PCR product after digestion with MspI, 6) C. dubliniensis PCR product after digestion with BlnI, M) Size marker 100bp.

4. Discussion

Vulvovaginal Candidiasis is a common vaginal infection affecting about 75% of women during reproductive age. The overall prevalence of Vulvovaginal Candidiasis in a community setting was found to be 58%. This was higher than in Hamadan (Shobeiri and Nazari, 2006), northwest of Iran and elsewhere and lower than Tehran (Pakshir et al., 2007). Some previous reports rated prevalence between 30 and 50 percent (Ross et al., 1995). *C.albicans* is the main cause of the disease with a prevalence of approximately 80-90% (Infections and Wong, 2006). Identification of *Candida* species by using traditional methods such as direct examination microscopy, culture, assimilation and fermentation of sugars is time-consuming and sometimes not cost-effective (Kazemi et al., 2013). Moreover, the obtained result may be difficult to interpret. In contrast, molecular biology techniques provide alternative methods and are becoming routine tools in the identification of yeasts (Sousa and Pereira, 2013). Each *Candida* species possesses unique nucleotide sequences that distinguish it from every other organism on the basis of the number and size of the fragments.

RFLP-PCR is based on the digestion of amplified DNA. Restriction analysis of the rDNA region spanning the 5.8S rRNA gene and flanking internal transcribed spacers (ITS_1 and ITS_4) has previously been shown to be an effective, rapid and simple method to identify a variety of yeasts isolated from clinical samples (Cirak et al., 2003). PCR-RFLP method has potential advantages in terms of speed, efficiency and reduced work load. In the present study, the frequency of isolated yeasts from Vulvovaginal Candidiasis was studied by PCR-RFLP method previously described by Mirhendi et al (2008) (Mirhendi et al., 2005). Consistent with other studies conducted in other countries, the predominant species isolated from vagina was *C.albicans*. Moallaei et al. (2011) was reported 62 cases of VVC out of 231. The isolated species were identified as follows: *C.albicans*, 24 (38.7%), *C.glabrata*, 15 (24.2%), *C.kefyr*, 13 (21.0%) *C.krusei*, 9 (14.5%), and *Saccharomyces cerevisi*ae, 1 (1.6%). Diba et al. (2014) reported 90 cases of VVC out of 192 suspected patients in which 11 cases suffered from RVVC. The *Candida* species identified including *C.albicans* (78.8%), *C.glabrata* (7.7%), *C.parapsilosis* (6.6%), *C.tropicalis* (4.4%) and *C. krusei* (2.2%) (Diba et al., 2014). Generally, *C.albicans* is still the predominant species of *Candida* isolated from patients with any kinds of candidiasis. In this study, two closely related species e.g. *C.albicans* and *C.dubliniensis* was successfully distinguished from each other by using *Bln*I restriction enzyme. Despite

that these two *Candida* species behave as one species in many physiological and molecular tests; they can be identified from each other using *Bln*I. There are few published data in which *C.dubliniensis* was distinguished from *C.albicans* in Iranian patients. Salehi et al. (2012) performed antifungal susceptibility testing for *Candida* species against eight antifungal drugs. The frequency of *Candida* species isolated from vaginal infected patients were identified as C.*albicans* 53 (79.1%), *C.glabrata* 8 (11.9%), *C.tropicalis* 4 (5.9%) and *C.krusei* 2 (2.9%). Highest sensitivity of *C.albicans* to antifungal drugs was seen against Miconazole (49 of 53 isolates) followed by, Clotrimazole [41], Terbinafine [28] and Ketoconazole [13] whereas 43 isolates were resistant to Fluconazole and Econazole (Salehei et al., 2012). Adesiji et al. (2011) demonstrated that 26 (25%) of the females (out of 104) had Vulvovaginal *candidiasis* with a species distribution of *Candida* isolates accounting for 13 (50%) with C.*albicans*, 6 (23%) with C.*glabrata*, 1 (4%) with C.*krusei*, and 6 (23%) with C.*tropicalis*. They were assay of the given fungi to the number of antifungal agents preparations used revealed the following: in fluconazole, 2 (7.8%) isolates were sensitive, 5 (19.2%) were dose dependent, and 19 (73%) were resistant. For Voriconazole, 10 (38.5%) isolates were sensitive, and 16 (61.5%) were resistant. For Nystatin, 15 (57.7%) isolates were sensitive and 11 (42.3%) were resistant (Adesiji et al., 2011). Quindos et al. (1999) showed that 90.2% and 91.4% of isolates of *Candida* species were sensitive to Fluconazole and Ketoconazole, respectively (Quindós et al., 1999). Andreu et al. (2001) isolated 68 *Candida* strains from vaginal smears. *C.albicans* represented 75% of the total strains whereas *C.parapsilosis*, *C.krusei* and *C.glabrata* were much less frequently found (Andreu et al., 2000). They evaluated minimum inhibitory concentration (MIC) for Nystatin against *C. albicans*. The obtained mean of MIC was 4mg/mL due to two strains that showed the highest MIC values (8 mg/mL). According to the published data, there is an increasing trend in susceptibility of *Candida* species against antifungal agents. As we also report here, 5 (13.89%) and 4 (11.11%) isolates of *C.albicans* strains were resistant to fluconazole and itraconazole, respectively.

In conclusion, *C.albicans* is still the predominant species causing *Candida*-related infections. Nevertheless, the number of isolated yeasts from other species is growing gradually. Identification of the causative agent to specie level is very important due to the increasing trend in the number of *Candida* isolates which have high MICs against antifungal agents.

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