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Identification of *Candida* species isolated from Iranian women with vaginal candidiasis by PCR-RFLP method

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ABSTRACT

The purpose of this study was to determine the occurrence of candida species in women referred to gynecology clinics (Tehran, Iran) with symptoms of vaginal candidiasis.150 vaginal swabs were collected from women with vaginal candidiasis. Then samples were cultured on Sabouraud Dextrose Agar for morphologic analysis. Genomic DNA of all clinical samples were extracted by glass bead and lysis buffer. PCR was performed to amplify ITS1-5.8SITS2 in candida genome using universal primers. For final identification of candida species the PCR-RFLP was done. The result of this study showed that there were candida species in vagina of 110(73%) of the patients and four species of candida were identified. By using molecular method including: Candida albicans(87.2%), Candida labrata(10%), Candida tropicalis(1.8%) and Candida parapsilosis. (1%). According to our results however C. albicans was the most frequently isolated species (82.2%), nowadays the occurrence of the other non-albicans isolates such as Candida glabrata is increasing in vaginal candidiasis. The identification of candida species in vaginal candidiasis due to developing antifungal resistance is very significant for appropriate treatment and to prevent the spread of recurrent candidiasis in women.

Key words: Vaginal candidiasis, *Candida* species, PCR-RFLP

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a mucosal infection caused by *Candida* species which is one of the most common clinical disorder in women of reproductive age[1,2]. The Colonization and adherence of *candida* species in epithelial cells are the first step in initiating of infection and creating vulvovaginal candidiasis. *Candida* species has some adhesion molecules such as *ALS*(agglutinin like sequence) and *HWP1* in attachment to host cell surface[3,4]. Moreover *Candida albicans* is the most frequent colonizer and responsible for vaginal candidiasis ,the various species of *candida* such as *C.glabrata, C.krusei, C. tropicalis and C.parapsiolsis* must be considered in vaginal candidiasis[5,6]. It is estimated that 75% of women will experience at least one episode during their life and 50% of them experience multiple episodes as well.

The incidence of vulvovaginal candidiasis is the highest rate in 20–40 year old women[7] .The prevalence of non-*albicans* species is increasing in vaginal candidiasis. The aim of the present study was identifying of *Candida* species occurrence in reproductive women by using RFLP-PCR assay. One of the most important reasons for distinguishing the *candida* species is that non-*C. albicans* species especially *C.glabrata* may be resistant against standard azoles therapy. [8].The classification of species and azole sensitivity should be operated in addition to

culture of them. The identification of *candida* species is very crucial Since *C. glabrata* is the most common 'non *albicans*' species with a poor response in vitro to fluconazole and using as the first line of treatment ,also gynecologists advise short courses of azole therapy [9]. for patients and up to 5% of women with vaginal candidiasis will have recurrent infections. Traditional methods such as morphological ,biochemical analysis colony morphotyping and serotyping are used for identification and typing of clinical *Candida* isolates, but these techniques are time consuming and dependent on phenotypic expression, that makes them potentially unreliable[10]. An alternative method of identification could be genotype-based one. Genotypic methods have been used extensively in detection and typing of *Candida* strains, [11,12]. In this study we used PCR-RFLP method to identify the *candida* species which isolated from women with vaginal candidiasis.

MATERIALS AND METHODS

Samples Collection and culture:
Women with signs and symptoms of VVC were studied. About 150 Vaginal sampling of the participants which admitted to Gynecology and obstetrics clinics (Tehran, Iran) during a one-year period from September 2011 to September 2012 ,collected by using a sterile swab. All of the patients signed a written consent form before participating in the study and patients' were strictly protected. Moreover, no difficulty was seen after taking the vaginal samples.

Samples were submitted to medical mycology Laboratory in Tarbiat Modares University and cultured on Sabouraud Dextros agar containing Chloramphenicol (Merck, Germany) to prevent growth of bacteria and incubated for 2 days at 35°C. After this time *Candida* infection was proved by microscopic observation of *Candida* pseudohypha or filamentous blastoconidia in direct preparations. This microscopic feature could be a probable reason for pathogenic form of *Candida* in vaginal tissue.

Molecular identification
All isolates of *Candida* species were examined by molecular method of PCR-RFLP, as following steps.

DNA extraction : Genomic DNA from clinical isolates and standard species of *candida* were extracted by glass bead and lysis solution according to previous described method [13] . briefly about 10 mm3 of a fresh colony was transferred to a 1.5 ml eppendorf tube and then 300 µl of lysis buffer containing(100 mM Tris pH 8, 10 mM pH 8, 100 mM NaCl, 1% SDS, Triton 2% X-100), 300 µl of phenol: chloroform (1:1) and 200 µl of glass beads, with a diameter of 1 mm, were added and the tube was vigorously shaken for 60 minutes, the sample was centrifuged for 5 minutes at 5000 rpm. The supernatant was transferred to a clean tube and 400 µl of chloroform was added. After centrifuging as the previous conditions, the aqueous phase was transferred to a clean tube and then 1 volume of cold isopropanol and 5 of 3M sodium acetate (pH: 5.2) were added and was kept at -20 °C for 10 minutes. After that, the sample was washed by 70% ethanol. Then 30 µl distilled water was added and the sample was kept at -20 °C.

PCR amplification: PCR was performed to amplify ITS1-5.8SITS2 segment in ribosomal DNA. For this, ITS1-5.8S-ITS2 universal primers were used. The sequences of ITS1 and ITS2 were 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively. PCR reaction was performed with the following components: 2.5 µl of 10x PCR buffer, 1.5mM MgCl2, 0.5 µl of 10 mM dNTPs, 0.4µM Primers, 1.25 units of Taq polymerase (Sinagene, Iran), 1 µl of template DNA and molecular grade dH2o up to 25 µl. The reactions were performed in a Thermo cycler (Bio Rad). Thirty amplification cycles were performed in the thermo cycler (Bio Rad) after initial DNA denaturation at 95 °C for 5 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 5 min following the last cycle.

Identification of *Candida* species using Restriction Fragment lengths Polymerase Assay (RFLP):
RFLP differential pattern were used in order to distinguish the *Candida* isolates. Restriction enzyme *Msp*I (Fermentas) were used for cutting the amplified DNAs of *Candida* spp .Digestion of amplified ITS fragments produced different size fragments for *Candida* species. For digestion, 10 µl of each PCR product was directly digested with by 5 U (1 µl) of the restriction enzyme *Msp*I, 1.5 µl of the digestion buffer, and dH2o up to 13 µl incubated at 37°C for 180 min. The digested fragments electrophoresed through 1.8% agarose gel and then visualized by ethidium bromide staining [14].

RESULTS

During twelve months from September 2011 to September 2012 totally 150 samples were collected from the patients with average 33 year old and having vaginal candidiasis symptoms.

Molecular assessment demonstrated that 110(73%) of the patients had *candida* spp infection.PCR products were given 500 to 600 bp fragments by using ITS1 and ITS2 primers and revealed in a 1.5% agarose gel electrophoresis after staining in 0.50 mg/ml of ethidium bromide . The results of the analysis of PCR-RFLP method showed *C. albicans* was the most frequently isolated species (87.2%), followed by *C. glabrata* (10%), *C. parapsilosis (1%)* and *C. tropicalis* (1.8%). According to our result, there was a significant correlation *(P* < 0.05) between the age of Patients and recognition rates of *C. albicans* infection, since by increasing the age of patients , other species of *candida* were more noticeable in vagina infections.

(Fig.1), shows PCR and PCR-RFLP patterns of different *Candida* spp**. (**Table 1), indicates the size of PCR product with ITS1-ITS2 primers for different standard species of *Candida* before and after restriction digestion with *MspI*.

Table 1: Sizes of ITS1-ITS2 PCR product for 6 *Candida* species, Before and after digestion by the restriction enzyme *Msp*I.

Candida species	*size of ITS1-ITS2(bp)*	*size of MspI digestion(bp)*
C. albicans	535	297, 338
C. glabrata	871	557, 314
C. tropicalis	524	340, 184
C. krusei	510	261, 249
C. parapsilosis	520	520

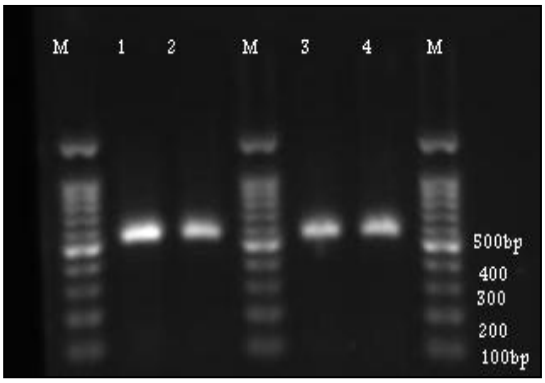

Fig1A PCR products of ITS1-F/ITS2-R amplification in *Candida parapsiolosis*
Line 1; C. parapsiolosis ATCC 90018, line2; clinical isolate of C. parapsiolsis, PCR-FRLP products of Candida parapsiolosis with MspI., line3 and 4; C. parapsiolosis isolated from patients. M; 100bp DNA ladder.

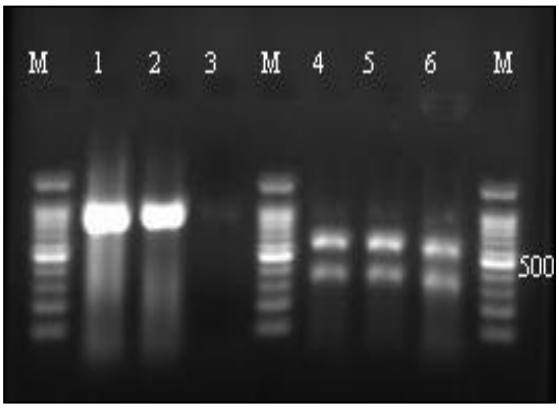

Fig1B PCR products of ITS1-F/ITS2-R amplification in *Candida glabrata*
Line 1; C. glabrata ATCC90030, line2; clinical isolates of C. glabrata, line3; Negative control. PCR-FRLP products of Candida glabrata with MspI. line4, 5 and 6; C.glabrata isolated from patients. M; 100bp DNA ladder

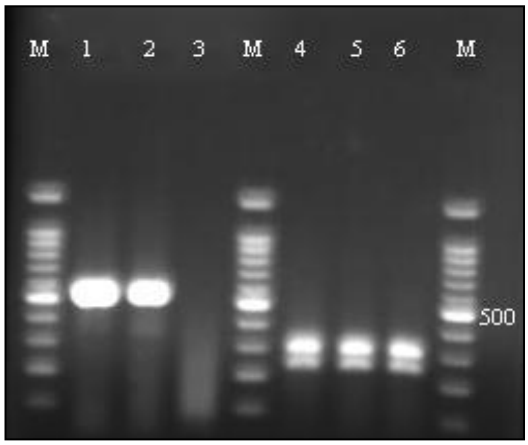

Fig1C PCR products of ITS1-F/ITS2-R amplification in *Candida albicans*
Line 1;C.albicans ATCC10231,line2; clinical isolates of C.albicans, line3;Negative control. PCR-FRLP products of Candida albicans with MspI.line4,5 and 6; C.albicans isolated from patients. M;100bp DNA ladder

DISCUSSION

Vaginal candidiasis (VC) is caused by over grows of broad spectrum of *candida* species in vagina with special symptoms including abnormal itching, vaginal discharge and burning sensation. Candida vulvovaginit is an important health compliant for gynecologist all over the world.

Vaginal candidiasis is routinely diagnosed without laboratory test, which results in misdiagnosing. This study was set to investigate prevalence VC among women attending gynecology and midwifery centers in Tehran, Iran. The age of the patients were between 25 - 42 . Although VC is treatable , without appropriate treatment , there is a probable risk for gaining of HIV/AIDS with other complications [15] including pelvic inflammatory disease, infertility, ectopic pregnancy. It is now well recognized that the occurrence of infective vaginal discharge seriously cause transmission and acquisition of HIV between sexual partners[16]. Therefore, the early diagnosis,prevention and prompt treatment of this problem is required. The ability of molecular biology methods to detect fungal pathogens is more reliable than traditional phenotyping methods[17,18]. Recently a variety of molecular biology methods were performed for the genetic identification of *Candida spp*. Some of these methods including standard PCR, multiplex PCR, PCR-RFLP, Real–time PCR, randomly amplified polymorphic DNA (RAPD)–PCR and DNA sequence analysis[19] . In this research, we recognized *Candida spp* using PCR-RFLP method by two universal primers ITS1 and ITS2 and the restriction enzyme *Msp*I.It which showed this technique is rapid, sensitive, and reliable with simple component of PCR; this method can be also used in clinical laboratories to identify clinically important fungi such as *Candida spp*. According to our finding *C.albicans* (87.2%).was the most yeast that isolated from vagina followed by *C. glabrata* (10%), *C. parapsilosis (*1%*)* and *C. tropicalis* (1.8%). Our result indicated that although *C. albicans* is the most species in this population, the other ones especially *C.galabrata* should not be ignored , because this species was the second element in women at older age, that may be result from changing in the level of hormone or other factors affected on vagina environment. Because of the increase in antifungal resistance and recurrent candidiasis, the significance of emerging non *albicans* species is very obvious[20,21]. Also our result is comparable with other studies which were done worldwide. Mahmoudi Rad et al identified *candida* species in women associated with vaginal candidiasis using multiplex PCR. they reported that *C.albicans* and then *C. glabrata* are the two most common causes of vulvovaginal candidiasis in this population[22]. Nowadays *Candida glabrata* is the most commonly reported non C.*albicans* species[23]. *C. glabrata* is now frequently identified in vaginal candidiasis with producing severe systemic mycosis and candidemia in critical and immunocompromised patients [24].

The genome of *C.glabrata* encode many of adhesions components for attachment to surface of host cells especially vagina, therefore this process is essential for initiation of infection and biofilm formation that causing antifungal resistant in this species [25].

Adherence factors and hydrophobicity of *C.glabrata* in comparison with other species is four times higher [26].

In immunocompromised patients especially people with Type II diabetes, rate of infections with *C. glabrata* is increasing. Treatment of relapsing forms of VVC in individuals suffering with diabetes, is very difficult or impossible which must be noticed by gynecologist[27]. *C.glabrata* is the second most frequently identified species (after *C. albicans*) in women with vaginitis having a prevalence between 0.6% and 36% with a mid-frequency

between 15% and 20%[28]. It has been confirmed that *C. glabrata* resists intrinsically against several antifungal drugs such as Azol, also the efficacy of some antifungal drugs which used in clinical therapy is low[29,30,31].Therefore, in comprehensive outlook the authors suggest the accurate and early diagnosis of vaginal candidiasis in order to achieve an appropriate treatment strategy which is desirable for clinicians.

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REFERENCES

[1] Sobel DJ, Faro S, Force R, Fox B , *Am j Obstet Gynecol*, **1998**,178,203-11.
[2] Haefner H , *Clin Obstete Gynecol*,**1999**, 42,184-95.
[3] Hoyer LL, Green CB, Zhao X ,*Medical Mycol*,**2008**, 46,1-15.
[4] Argimon S , Wishart JA, Leng R , Macaskill S , Mavor A, Alexandris T, *Euk Cell* ,**2007**, 6, 682-692.
[5] Barousee MM, Vanderpol BJ, Fortenberry D, Orr D, Fidel PL, *Sexual Trans Infect*,**2004**, 80,48-53.
[6] Ridgway GL, *Sexual Trans Infec*,**2000**,76,147.
[7] Mardh PA ,Rodrigues A , Genc M, Novikova N, Martinez-de-Oliveira J, Guaschino S, *Inter J STD AIDS*, **2002**,13,522-39.
[8] Mardh PA, Novikova N, Witkin S, Korneeva I, Rodriques AR, *Inter J STD. AIDS*,**2003**, 14,753-6.
[9] Fidel PL, Vazqluez J, Sobel JD , *Clin Microbiol Rev*,**1999**, 12,80-96.
[10] Williams DW, Wilson M, Lewis M, Potts A, *J Clin Microbiol* ,**1995**,33(9),2476-9.
[11] Buchman T, Rossier M, Merz W, Charache P, *Surgery*,**1990**, 108,338-46.
[12] Elie E , Lott TJ , Reiss E , Morrisson CJ ,*J Clin Microbiol*,**1998**, 3260-5.
[13] Yamada Y , Makimura K, Merhendi H, Ueda K, Nishiyama Y , *J Infec Dis*,**2002**, 55,122-5.
[14] Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi H, *J Med Microbiol*,**2006**,47,225-9.
[15] Kenneth O , *J Genito Med*,**2003**,18,18-30.
[16] Abebe E, Olumide M, Oke O,*Federal Ministry of Health Abuja*,**2001**, 3-7.
[17] Shokohi T, Hashemi Soteh M, Pouri ZS, Hedayati M, Mayahi S,*Inter J Med Microbiol*,***2010***, 28(2),147-51.
[18] Shokohi T, Bandalizadeh Z, Hedayati MT , Mayahi S, *J J Microbiol*,**2011**, 4,19-26.
[19] Yamada Y, Makimura K, Uchida K, Yamaguchi H, Osumi M, *Mycoses*,**2004**, 47:24-8.
[20] Abu-Elteen KH, *J J Infec Dis*,**2001**, 54(3),103-7.
[21] Bauters TG, Dhont MA, Temmerman MI , *Am J Obstet Gynecol*,**2002**,187,569-74.
[22] Mahmoudi Rad M, Zafarghandi A , Zabihi MA, *Infec Dis in Obstet Gynecol*,**2012**,1-5.
[23] Nwadioha SI, Egah DZ, OAlao O , Iheanach E, *J Clini Med Research*,**2010**,2(7).
[24] Weig M, Jansch L, Gross U, Koster CD, Klis F, Groot PD, *Microbiol*,**2004**, 150,3129-44.
[25] 25 .Mundy R, Cormack B, *J Infect Dis*,**2009**,199,1891-8.
[26] DeLas Penas A, Pan SJ, Castano I, Alder J, Cregg R, Cormack B, *Genes and Develop*,***2003***,17,2245-58.
[27] Figueroa R, Santibáñez JA, Kuba E, Trujillo A,*Cirugia Y Cirujanos*,***2009***, 77(6),423-7.
[28] Fidel P , Vazquez JA, Sobe JD,*Clin microbial rev*,**1999**,12(2),80-96.
[29] Goffeau A, *Nature*,**2008**,452,541-2.
[30] Cannon RD, Lamping E, Holmes AR, Niimi K, Tanabe K, Niimi M, *Microbiol*,**2007**, 153:3211-7.
[31] Odds F, Brown A, Gow NA, *Trends Microbiol*,**2003**, 11,272-9.