Advances in Bioresearch
Adv. Biores., Vol 7 (6) November 2016: 40-43
©2016 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV Value 8.21 [2014]

Advances in Bioresearch

ORIGINAL ARTICLE

Use of PCR-RFLP for Molecular Identification of *Candida* species isolated from Vulvovaginal candidiasis

Elham Baghdadi[1], Fatemeh Noorbakhsh[1], Hadis Kalantari Moghaddam[2], Sadegh Khodavaisy[3,4], Sassan Rezaie[*4]

[1]Department of Microbiology, Islamic Azad University, Varamin-Pishva Branch, Iran
[2]Master of Sciences in Microbiology, Environmental Research Station, Anzali, Iran
[3]Department of Medical Parasitology and Mycology, Kurdistan University of Medical Sciences, Sanandaj, Iran
[4]Division of Molecular Biology, Department of Medical Parasitology and Mycology, Faculty of Public Health, Tehran University of Medical Sciences, Tehran, Iran
*Corresponding Author's E-mail; srezaie@tums.ac.ir

ABSTRACT

Vulvovaginal candidiasis is a common mocusal infection in genital tract among women which up to 75 percent of women may experience this disease at least once in their lifetime. Due to the high degree of phenotypic similarity between Candida species, identification problems are imminent. Rapid and accurate diagnosis of causative agents of vulvovaginal candidiasis with molecular techniques is necessary for epidemiological purposes and for effective treatment.The aim of this study is analysis of frequency of candida species in vulvovaginal candidiasis patients based on molecular approaches.Fifty isolates from patients with suspicious symptoms of VVCinitially were identified by phenotypic methods andconfirmed by molecular approaches based on PCR-RFLP.27 (54%) species of C.albicans,12 (24%)species of C.glabrata, 6 (12%) species of C.krusei, 3 (6%) species of C.kefyr, 2 (4%) species of C.tropicaliswere identified as pathogen yeasts.Like the most of similar studies performed in this field, the present study found Candida albicans as common species isolated from vulvovaginal candidiasis. PCR-RFLP is rapid, sensitive, and reliable that might be also used for other similar epidemiological studies and medical mycology laboratories.

Keywords: *Vulvovaginal candidiasis, Candida species, PCR-RFLP*

Received 25/03/2016 Accepted 21/07/2016

How to cite this article:
E Baghdadi, F Noorbakhsh, H K Moghaddam, S Khodavaisy, S Rezaie. Use of PCR-RFLP for Molecular Identification of *Candida* species isolated from Vulvovaginal candidiasis. Adv. Biores., Vol 7 [6] November 2016: 40-43. DOI: 10.15515/abr.0976-4585.7.6.4043

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a common mocusal infection in genital tract among women which are in reproductive age[1]. VVC occurs by overgrowth of candida yeasts in vagina [2]. *Candida albicans* is the major agent of VVC which cause 80-90 percent of vaginal infections [3]. Up to 75 percent of women may experience vulvovaginal candidiasis [1,4]. Incontrast, approximately 5-10 percent of the patients suffer from recurrent VVC which is complicated form of vaginitis [5,6]. Recently *non-albicans* species play an important role in VVC, such as *C.glabrata, C.krusei, C.tropicalis, C.parapsilosis* that have shown resistance to azoles [7]. VVC occurs in HIV infected, cancer patients, immunocompromised persons or those with diabetes mellitus [8,9,10].Diagnosis of *candida* species by conventional morphologic characterization can require three to five days. Thus it is nessesary to identify the pathogenic fungi at species level for achievement of appropriate clinical treatment [11]. Molecular techniques are useful because of their specificity and sensitivity [9]. A specific method to identify Candida yeast species is based on the use of primers to obtain ITS1-5.8S-ITS4 gene fragment by PCR [12]. The Internal Transcribe Spacer regions in ribosomal gene have been used for primer designing [13]. There are different molecular methods for diagnosis of yeasts and *Candida* species such as using specific primers in Polymerase Chain Reaction and Multiplex PCR [14,15], Real time PCR[16], Sequencing of gene fragments[17], Restriction Fragments

Length Polymorphism (RFLP)[18,19]. RFLP is an easy, nonexpensive and rapid method and it only needs standard molecular equipment [13]. The aim of this study is analysis of frequency of *C.albicans* in vulvovaginal candidiasis patients based on genotypic approaches.

MATERIAL AND METHODS

Fungal isolates:

Fifty samples collected from women with suspicious symptoms of VVC from Medical Mycology Laboratory of Tehran University, Iran. All isolates were cultured on Sabouraud Dextrose agar with chloramphenicol and incubated at 37˚C for 48 hours. Primarily, these strains were initially identified by phenotypic and physiologic criteria, i.e., colony color on CHROMagar Candida medium (CHROMagar Company, Paris, France), germ-tube tests in serum at 37 ºC for 2–3 hours, microscopic morphology on Corn-meal agar (CMA, Difco)with 1% tween80. One colony on each identification strains was sub-cultured for molecular investigation.

DNA extraction:

For DNA extracting 300 µllysis buffer, 300 µl PCI and 300 µl glass beads (0.5 mm in diameter) added to collected yeasts. Yeast cells were broken by shaking the tubes for 5 min. Samples were centrifuged at 5000 rpm for 5 min, then the aqueous layer were transfered to new tubes. 300µl CI was added and centrifuged at 5000 rpm for 5 min again. Higher layer was transfered to a new tube and ad equal volume of Isopropanol and 0.1 volume of sodium acetate was added and tubes were vortexed. After incubation at -20˚C for 10 min. samples were centrifuged at 12000 rpm for 12 min. The supernatant was taken out and the present pellet was washed with 300 µl 70% ethanol. After centrifuging at 5000 for 5 min, the pellet was air-dried and solved in 50-100 µl distilled water. Samples were kept at-20˚C until use.

PCR- RFLP analysis:

PCR amplification of ITS1-5.8S-ITS2 rDNA regions was achieved using the ITS1 (forward, 5' -TCC GTA GGT GAA CCT GCG G-3' and ITS4 (reverse, 5' -TCC TCC GCT TAT TGA TAT GC-3') primer pairs To amplify ITS domains, PCR amplification was performed in a final volume of 50 µl. Each reaction consists of 2 µl template DNA, 0.5 µl of each primer at 25 µM, 1.25 µl of dNTP at 5 mM, 0.5U Taq DNA polymerase and 5 µl 10× PCR buffer. The amplification parameters consist of 35 cycles of denaturation at 94°C for 1 min, primer 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. Amplified products were visualized by 1% agarose gel electrophoresis in TBE buffer (20 mmol/l EDTA, 10 mmolTris boric pH 8). Gel was stained with Ethidium bromide (0.5 µg/ml) and photographed by ultraviolet photography.

Restriction Enzyme Analysis:

For PCR-RFLP technique, the restriction sites of various restriction enzymes were determined by CLC workbench software andMsp1 enzyme(Roche diagnostics, Swiss) was selected to digest the PCR products. The enzyme *MspI* makes DNA cleave where there is a CCGG sequence [10]. Digestion was performed by incubating 2µl of 10X buffer, 2 µl of Msp1, and 10 µl of PCR productin a final reaction volume of 10µl at 37ºC for 3 h. The resultant fragments were electrophoresed through 1% agarose gel stained with ethidiumbromide(0.5 µg/ml). The digested fragments visualized under UV transluminator and photographed. The size of DNA fragments distinguished directly with comparison of molecular size marker and separated banding patterns which indicated in relevant studies.

Sequence analysis of ITSr DNA region:

Randomly for validation the identity of the species sequenced. Sequence data were aligned using MEGA 5.05 (http://www.megasoftware.net/) software packages and compared with GenBank database using the BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

The ITS regions of all samples were completely amplified. Electrophoresis of PCR products showed 535 bp band associated with *C.albicans*, 870 bp band associated with *C.glabrata*, 510 bp band for *C.krusei*, 720 bp band for *C.kefyr* and 524 bp band for*C.tropicalis*(Fig1).After the digestion of ITS region by Msp1 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*; 261, 249 for *C. krusei*.Results show two bands for all species except*C.kefyr*.The banding patternswere distinguishable and computable to recognize *Candida*species(Fig1). Results show that *C.albicans* was the most frequently isolated species (54%), follow by *non-albicans* included *C.glabrata* (24%), *C.krusei* (12%), *C.kefyr* (6%), *C.tropicalis* (4%) (Table 1).

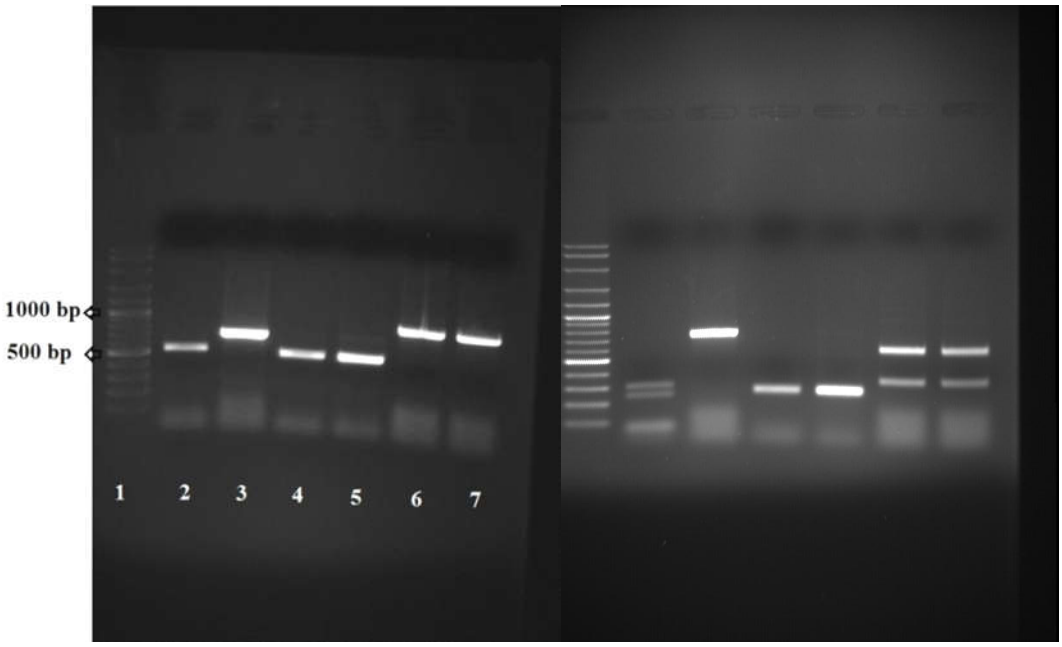

Figure1 (Left) PCR products of ITS1/ITS4 amplification in *Candida* spp. (Right) Patterns of PCR products of *Candida* isolates after digestion by the restriction enzyme *MspI*. Lane 1 is 100 bp molecular size markers; 2,*C.albicans*; 3, *C.kefyr*; 4&5*, C.krusei*; and 6 & 7, *C.glabrata*.

Table1; Frequency of *Candida* species

Candida species	Number	Frequency
C. albicans	27	54%
C. glabrata	12	24%
C. krusei	6	12%
C. kefyr	3	6%
C. tropicalis	2	4%

DISCUSSION

In this study the prevalence of Candida species was investigated by PCR-RFLP method. Consistent with the results of previous studies,*C. albicans*was the most common species(54%),in patients with vulvovaginal candidiasis. Rapid diagnosis of Candida yeasts in VVC patients is very essential for begining ofclinical treatment. *Non-albicansCandida* spp. such as *C. glabrata, C. krusei, C. tropicalis, C. kefyr* and *C. parapsilosis* have shown resistance to antifungal drugs, thus an effective treatment should be done. Due to time-consuming of phenotypic methods, molecular techniques have increased for detection of Candida in VVC [20]. However there are different methods to speciate*Candida* isolates such as DNA sequencing, RAPD, real time PCR, but RFLP considers as a simple and reliable method which does not need expensive equipments [13,20]. Our finding revealed that *C.albicans*as predominant species, followed by *C. glabrata*(24%), *C. krusei* (12%), *C. kefyr*(6%) and *C. tropicalis* (4%)isolated from VVCpatient. In a similar study by Mirhendi et al in Iran, C. albicans had the most frequency among other species[10], also in another study by Shokohi*et al. C. albicans* was the most species [18]. In a similar research by Noumi*et al*. that was performed by RCR-RFLP, *C. albicans* was the most yeast[12]. RFLP technique has been used by Farasat et al to identify *Candida* yeasts too [19]. In a research by Carvalho *et al* Multiplex PCR was described for identification of eight clinically relevant *Candida* species [15]. Real time PCR assay was analysed for detection of fungi agents [16]. Multiplex PCR has low specificity and sensitivity to define fungal agents and fewer fungi can be detected with this technique furthermore Multiplex and Real-time PCR needs expensive and developed equipments[13]. ITS1-5.8-ITS2 region in rDNA is a conserved sequence and after PCR, amplicons are made with variable length for fungi especially *Candida* species [9,21].

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 should be performed.

REFERENCES

1. Fan SR, Bai FY, Liao QP, Liu ZH, Li J, Liu XP.(2008). Genotype distribution of *Candida albicans* strains associated with different conditions of vulvovaginal candidiasis, as revealed by microsatellite typing. Infect; 84(2): 103-6.
2. Rathod SD, Klausner JD, Krupp K, Reingold AL, Madhivanan P. (2012). Epidemiologic Features of Vulvovaginal Candidiasis among Reproductive-AgeWomen in India. Infectious Diseases in Obstetrics and Gynecology. 1-8.
3. Sobel JD.(2007). Vulvovaginalcandidosis. Lancet; 369(9577): 1961-71.
4. Marot-Leblond A, Nail-Billaud S, Pilon F, BeucherB,DanielPoulain, RobertR. (2009). Efficient Diagnosis of Vulvovaginal Candidiasis by Use of a New Rapid Immunochromatography Test. Journalof Clinical Microbiology; 47(10): 3821–3825.
5. Paul L, Fidel Jr, Vazquez Ja, SobelJd. (1999).Candida glabrata: Review of Epidemiology, Pathogenesis, andClinical Disease with Comparison to C. albicans. Clinical Microbiology Reviews;12(1): 80–96.
6. Erika N, Ringdahl Md. (2000).Treatment of Recurrent Vulvovaginal Candidiasis. Am Fam Physician;61(11):3306-3312.
7. Khosravi AR, Shokri H, Kermani S, Dakhili M, Madani M, Parsa S.(2011). Antifungal properties of Artemisia sieberiandOriganumvulgare essential oils against Candida glabrata isolates obtained from patients withvulvovaginal candidiasis. Journal de MycologieMédicale; 21, 93-9.
8. Yi Ping Ge, Le Wang, Gui Xia Lu, Yong NianShen, Wei Da Liu.(2012).A simple and reliable pcr-restriction fragment length polymorphism assay to identify candida albicans and its closely related candida dubliniensis. Brazilian Journal of Microbiology: 873-879.
9. AyatollahiMousavi S.A, Khalesi E, ShahidiBonjar GH, Aghighi S, Sharifi F, Aram F. (2007). Rapid Molecular Diagnosis for Candida species Using PCR-RFLP. Biotechnology;6(4):583-587.
10. Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi H. (2006). A One-Enzyme PCR-RFLP assay for identification of six medically important Candidaspecies. Nihon IshinkinGakkaiZasshi;47:225-9.
11. Baquero C, Montero M, Sentandreu R, Valentin E. (2002). Identificationof Candida albicans by polymerase chain reaction amplification of aCaYST1 gene intron fragment. Rev. Iberoam. Micol; 19: 80-83.
12. Noumi E, Snoussi M, Vercher MP, Valentin E, Castillo LD,BakhroufA. (2010). Identification of Candida glabrata and Candidaparapsilosis strains by polymerase chain reaction assay using RPS0 gene fragment. Journal of Yeast and Fungal Research; 1(9) 170–173.
13. Santos MS, Souza ES, Junior RMS, Talhari S, Souza JVB. (2010). Identification of fungemia agents using the polymerase chain reaction and restriction fragment length polymorphism analysis. Brazilian Journal of Medical and Biological Research;43: 712-716.
14. Garcia Martínez M, del Castillo Agudo L. (2010). Identification ofpathogenic yeast species by polymerase chain reaction amplificationof the RPS0 gene intron fragment. J. Appl. Microbiol; 108(6): 1917-1927.
15. Carvalho A, Costa-De-Oliveira S, Martins ML, Pina-Vaz C, Rodrigues AG, Ludovico P, Rodrigues F. (2007). Multiplex PCR identification of eight clinically relevant Candida species. J.MedMycol;45(7):619-27.
16. Fricke S, Fricke C, Schimmelpfennig C, Oelkrug C, Schönfelder U, Blatz R, Zilch C, Faber S, Hilger N, Ruhnke M, Rodloff AC. (2010). A real-time PCR assay for the differentiation of Candida species.JApplMicrobiol;109(4):1150-8.
17. Odds FC, Jacobsen MD. (2008). Multilocus sequence typing of pathogenic Candida species.Eukaryot Cell;7(7):1075-84.
18. Shokohi T, Soteh MB, SaltanatPouri Z, Hedayati MT, Mayahi S.(2010). Identification of Candida species using PCR-RFLP in cancer patients in Iran. Indian J Med Microbiol;28:147-51.
19. Farasat A, Ghahri M, Mirhendi H, Beiraghi S. (2012). Identification of Candida Species Screened from Catheter Using Patients with PCR-RFLP Method. European Journal of Experimental Biology; 2(3):651-656.
20. Vijayakumar R, Giri S, JyotiKindo A. (2012). Molecular Species Identification of Candida from Blood Samples of Intensive Care Unit Patients by PolymeraseChain Reaction – Restricted Fragment Length Polymorphism. Journal of Laboratory Physicians; 4(1):1-4.
21. Berman J, Sudbery PE. (2002). Candida albicans: A molecular revolution built on lessons from budding yeast. Nature Rev;3:919-930.