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Identification of *Candida* species Isolated From Vulvovaginal Candidiasis Patients by Chromgen agar and PCR-RFLP Method

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Abstract:

This study focuses on diagnosis of *Candida* species causing Vulvovaginal Candidiasis using phenotype and genotype analyzing methods, and frequencies of candida species also using Vulvovaginal Candidiasis patients. 130 samples (100 from patients and 30 from non infected women) were collected and cultured on biological media. Identifying the yeasts, initially some phenotypic experiments were carried out such as germ tube**,** from motion of pseudohyphae and clamydospores in CMA+TW80 medium, API20 candida and CHROMagar Candida. Genomic DNA of all species were extracted and analyzed with PCR and subsequent Polymerase Chain Reaction - Restriction Fragments Length Polymorphism (PCR-RFLP) methods. Frequency of *C. albicans, C. krusei, C. tropicalis , C. parapsilosis and C. glabrata* were 46.4%, 31%, 18%, 7.2%, and 1.8%, respectively.The ITS1-ITS4 region was amplified and the Restriction enzyme *Msp*1 digests this region and was used to identify of *candida* species .Electrophoretically ribosomal DNA of *C. albicans, C. krusei, C. tropicalis* and *C. glabrata* produced two bands whereas the *C. parapsilosis* gave one band.

Key words: PCR-RFLP, *Candida albicans*, CHROMagar Candida, Vulvovaginal candidiasis

Introduction:

Vulvovaginal candidiasis (VVC) is an insidious that affects a large porportio in of women of all ages, and 5 to 8 of affected women experience recurrent VVC (RVVC) [1], and it is a common problem in women and may affect their physical and emotional health, as well as relationships with their partners [2]. There are two forms of RVVC: primary RVVC is idiopathic with unknown predisposing factors, secondary RVVC is the occurrence of frequent episodes of acute VVC because of certain predisposing factors such as hormone replacement therapy or diabetes mellitus [3]. VVC is caused by overgrowth of *Candida* yeast species in the vagina and is characterized by curd-like vaginal discharge, itching, and erythema[4]. *Candida albicans* remains the most

common cause of candidiasis, but other species are not uncommon[5]. *Candida albicans* account for 70 to 90 of all VVC cases, with a recent emergence of non-*albicans* species [6]. The rise in VVC infection, more specifically in those caused by non-*albicans* species, could be due to several factors, ranging from an increase in over- the –counter antifungal use to an increase in high-risk patient populations (i.e., diabetics and menopausal women). *Candida glabrata* is the primary non-albicans species emerging in VVC, accounting for up to 14% of infection in immune- competent women [6]. *Candida glabrata* was found to be the primary species isolated from diabetic (61.3%) and elderly (51.2%) patient with VVC [7]. The detection of *Candida* in vaginal swabs is corelated with the age of patients. It was shown that women under 35 years old have the highest rate of detectable *Candida* compared to the other groups, The detection rate of non-*albicans Candida* increased 2.75 folds in the age group of 26 to 35 years. [8] the incidence of VVC in pregnant women was 3.5 fold higher than that of non-pregnant women. It continued to increase in the third trimester of pregnancy [8]. Pregnancy has been known to be associated with depressed aspects of cell-mediated immunity that permit fatal retention. Moreover, the hormonal changed milieu of the vagina during pregnancy enhances *Candida* colonization and serves as a risk factor for symptomatic expression [9] .Delay in speciation of candida isolates by conventional methods and resistance to antifungal drugs (especially fluconazole, amphotericin B, etc.) in various *Candida* species are some of the factors responsible for the increase in morbidity and mortality due to candidemia. So, the rapid detection and Identification of Candida isolates is very important for the proper management of patients having candidemia [10].The RFLP-PCR using the restriction enzyme *MpsI* is a good rapid identification method that identifies the most important *Candida spp* isolated from patients and recommends further studies to develop new methods using different restriction enzymes to increase the range of identified *candida spp* [11].This study aims to focuses on diagnosis on *Candida* species based on phenotypic and genotypic approaches and analysis of frequency of Candida species in vulvovaginalcandidiasis patients.

Materials and Methods:

Patients:

One hundred of high vaginal swabs were taken from 100 married women, 30 of them were non – pregnant (N.P) and 70 were pregnant (P.) women. They were suffering from vulvovaginal candidiasis in addition to 30 healthy controls. Sample were taken during the period from first of June 2012 till the end of April 2013, under the supervision of specialized gynecologist in the bent Al- Huda hospital, Thi Qar. A special questionnaire was prepared for each individual.

The swabs were incubated in Sabouraud's dextrose agar (SDA) with chloramphenicol (0.5 mg/ml) at 37°C for 48 h .(under aerobic conditions) and in CHROMagar ™ *Candida* (CHRO Magar, France) at 35°C for 48 h for production of species-specific colors. Different chromogenic culture media are capable of distinguishing *C. albicans* from other clinically important yeast strains are commercially available. Such media distinguish *Candida* strains from other yeast strains on the basis of the color changes produced by the *Candida* colonies, which are measured using pH indicators and by fermentation of specific compounds or chromogenic substrates for the presumptive identification of *C. albicans*, *C. tropicalis,* and *C. krusei*[12]. 10% KOH preparation and Gram stain for

microscopic examination of pseudohyphae and yeast cell forms. Carbohydrate assimilation tests was used. Fresh yeast colonies were incubated with rabbit serum at 37°C for 3 h to test for germ tube formation.Development of filamentous-form cells and chlamydospore formation were evaluated by culturing the yeast isolates on Dalmau plates (cornmeal-Tween 80 agar) at 30°C for 48 h [13]. and identification using API 20 AUX (Bionereux, Paris, France). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed using specific primers for the molecular identification of *Candida spp*.

DNA extraction:

For DNA extraction, yeasts were grown on Sabouraud dextrose agar plates (Difco) at 37 °C for 24-48 hrs. A single colony was cultured overnight on YPD broth (1% yeast extract, 2% peptone, 2% dextrose) at 37 °C. DNA was extracted using the DNA Isolation Kit (BIO BASIC INC,Canada).

PCR amplification

PCR amplification of ITS1-5.8SITS2 rDNA regions were 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 (Fermentans, Germany) which were described previously (14,15). PCRamplifications were carried out in 25µl volumes containing 1 µl of each primer, 12.5µl of GoTaq Green Master Mix (Promega, Madison, WI, USA), 5µl DNA template and corresponding amount of ultra-pure distilled water. Amplifications were carried out in a thermal cycler (Perkin-Elmer cetus type 480). The amplification parameters consisted 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. Expected products of amplification are 510- 871 bp (*C.albicans* 535 bp, C *glabrata* 871 bp, *C trobicalis* 524 bp, C *krusei* 510 bp, C *parapslosis* 520 bp) [14].

Restriction enzyme analysis:

A volume of 25 µL of PCR products were digested directly and individually by the restriction enzyme *MspI* . For each restriction digestion reaction, 5 µL of the amplified PCR product was digested with 1.5 µL of restriction enzyme buffer, 0.5 µl of the restriction enzyme *Msp*I, and 8 µL of Deionizer distilled water; the reaction mixture 15 µL was incubated at 37°C for 120 min. Separation of the digested fragments was visualized on 2% agarose gel run in TBE buffer at 100 V for 45 min, and stained with 0.5 µg ml-1 ethidium bromide [15].

Results and Discussion:

Isolation of *candida spp*.

We identified the different *Candida spp.* from women infected with VVC and healthy women as control group, by using the restriction enzyme *Msp*I. (PCR-RFLP assay),chromogen agar, Biochemical tests and API20 *C. albicans* were the most commonly identified species (41.4 %), followed by *C.krusie* (31.5%)*C. tropicalis* (18%), *C. parapsilosis*(7.2) and *C.glabrata* (1.8%),while the species *C. albicans* , *C.krusie* and *C. tropicalis* recorded (16.66%,6.66%,3.33%) respectively in controle group as shown in table(1). This result agrees with AL-Hashime who isolated 60 isolate of *Candida albicans* from 120 Non-pregnant infected women with volvovaginal candidiasis [16] and with Roudbary *et al* who indicated that *Candida albicans* is the most dominant species compare with other species[17]. *Candida albicans* is the most abundant isolated microorganism from Volvovaginal Candidiasis patients with frequency of 47.2% [18].

Habibeh *et al*.[19] have shown that *C. albicans* 53.64% as the major causative agents of Vulvovaginal Candidiasis. This because *Candida albicans* have high ability to adherence on epithelial cells and its ability to produce germ tube in infected tissue ,and high product to protein digestive enzymes and phospholipase enzymes [20]. The color of *Candida albicans* colonies on CHROMagar *Candida* was green, while *Candida tropicalis* was blue and *Candida krusei* was pink fuzzy as in figure (1).

Table(1):The Frequency of Isolation of clinically important *Candida spp*. From femal GIT

Types of *candida*	Number of isolates			
	Healthy	%	VVC	%
Candida. albicans	5	16.66	46	%41.4
Candida glabrata	0	0	35	%.31
Candida tropicals	1	3.33	20	%18
Candida parapsilosis	1	3.33	8	%7.2
Candida krusie	2	6.66	2	%1.8

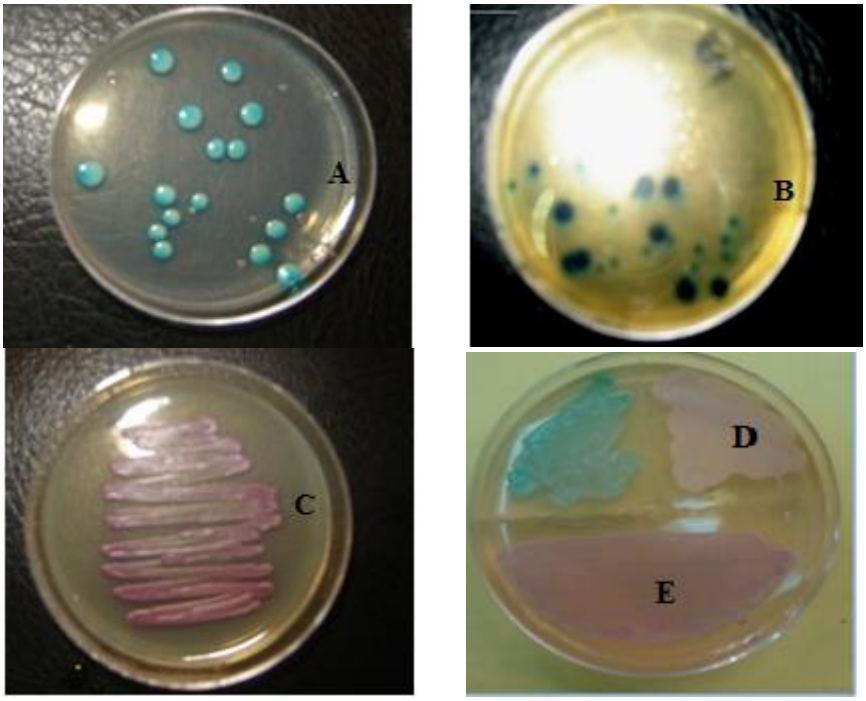


Fig. (1) show the *Candida spp* growing on chromogen agar at 37C°for 5 days (A: *C.albicans* , B: *C.tropicalis* , C: *C.glabrata* , D: *C.krusie*

The molecular characterization of *Candida spp*. was done on the basis of the number of digested DNA bands in the ITS region. The intergenic spacer region was successfully amplified from all *Candida* isolates tested giving amplification product 510-871 bp. Similar results were observed by Allam and Salem [11] and by Ayatollahi Mousavi *et al*., and Mirhendi *et al*., [21,14]. As illustrated in figure (2) PCR products of Approximately 535 bp for *C. albicans*, 520 bp for C. *parapsilosis* , 871 bp for *C. glabrata* , 510 bp for *C. krusei* and 524 bp for *C trobicalis* The molecular weight for *Candida* species are similar with those indicate by Mousavi *et al* and Vijayakumar *et al*. [21,10] *Candida albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis* are produced 2 bands whereas the *C.parapsilosis* showed 1band after digestion with *Msp*I. The size of the pre and post-digestion ITS1–ITS4 PCR products for *Candida spp*. are reported in *Table2*. The patterns obtained after *Msp*I restriction digestion of the PCR products of *Candida* isolates are shown in Figure(3).This result was in agreement with Ayatollahi Mousavi *et al* [22] methods, his experiment gave two

bands for each of *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* and three bands for *C. guilliermondii* (30).while in Allam and Salem [11]study gave 3 fragment for C *guilliermondii*, two fragments for *C.albicans*, *C krusei*, *C glabrata*, *C trobicalis* and *C stellatoidea*, and no effect on *C parapsilosis* amplicon. This isolate may have mutation in the recognition site of restriction enzyme *MspI*. Although it is a rare possibility but it can occur.

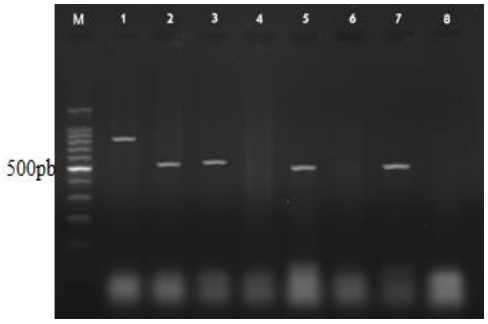
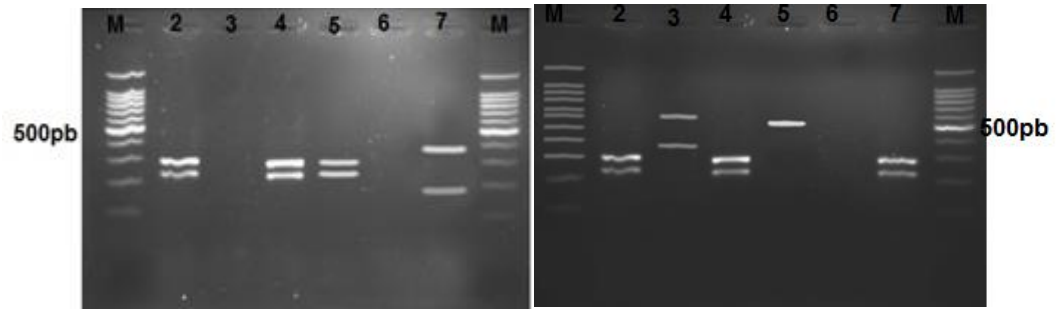


Fig. (2) The Patterns PCR Products of *Candida* Isolates Before Digestion With the Restriction Enzyme *Msp*I (molecular marker (M); lane1 *C glabrata*, lane 2 *C tropicalis*,lane 3*Candida albicans* Lane 5 C *krusei* and lane 7 *C parapsilosis.*



A B

Fig.(3)The Patterns PCR Products of *Candida* Isolates after Digestion With the Restriction Enzyme *Msp*I (A:lane 1,8: molecular marker (M); lanes 2and 4 *C.albicans* lane5 *C krusei* lane 7 *C tropiclis* (B:Lane 1,8: molecular marker (M); lanes 2, 4, and 7 .*Candida albicans* Lane 3 *C glabrata* lane 5 *C parapsilosis*).

Table 2 .The Size of ITS1-ITS4 PCR products for *Candida* spp. Before and After Digestion With Msp1

Candida spp	Size of ITS1-ITS4,bp	Size of Restriction product,bp
C albicans	535	297,238
C. glabrata	871	557,314
C. parapsilosis	520	520
C. krusei	510	261.249
C. tropicalis	524	340.184

References:

[1]Zhou, X.; Westman, R.; Hickey, R.; Hansmann, M.; Kennedy, C.; Osborn, T. and Forney L, 2009. Vaginal Microbiota of women with frequent vulvovaginal candidiasis, Infection and Immunity. Am. Soc. Microbial 77(9) P: 4130- 4135.

[2]Chapple, A.; Hassell, K.; Nicolson, M. and Cantrill, J. 2000. 'You don't really feel you can function normally: women's perceptions and personal management of vaginal thrush. J Reprod Infant Psychol; 18:309–319.

[3]Fidel, PL. 2004 History and new insights into host defense against vaginal candidiasis. Trends Microbiol 12: 220–227.

[4]Achkar, J. M. and Fries, B. C. 2010. Candida infections of the genitourinary tract, Clin. Microbiol. Rev, 23 (2): 253–273.

[5]Chang, HC.; Leaw, SN.; Huang, AH. Wu, TL and Chang, TC. 2001. Rapid identificacion of yeasts in positive blood cultures by a multiplex PCR method. J .Clin. Microbiol; 39: 3466-3471.

[6]Paulitsch, A.; Weger, W.; Ginter-Hanselmayer, G.; Marth, E and Buzina, W. 2006. A 5-year (2000–2004) epidemiological survey of

Candida and non- *Candida* yeast species causing vulvovaginal candidiasis in Graz, Austria. Mycoses 49:471–475.

[7] Ray, D.; Goswami, R.; Banerjee, U.; Dadhwal, V.; Goswami, D.; Mandal, P.; Sreenivas, V and Kochupillai, N. 2007. Prevalence of *Candida glabrata* and its response to boric acid vaginal suppositories in comparison with oral fluconazole in patients with diabetes and vulvovaginal candidiasis. Diabet Care, 30:312–317.

[8] Moallaei, H.; Mirhendi, S. H.; Mirdashti, R. and Rosado, R. 2011. Comparison of Enzymatic Method Rapid Yeast Plus System with RFLP-PCR for Identification of Isolated Yeast from Vulvovaginal Candidiasis, Iran J Basic Med Sci, 14 (5): 443-450.

[9] Xu, J. and Sobel, JD.(2004). Candida vulvovaginitis in pregnancy. Curr Infect Dis ; 6:445-449.

[10] Vijaykumar, R.; Giri, S. and Kindo, A J. 2012. Molecular Species Identification of Candida from Blood Samples of Intensive Care Unit Patients by Polymerase Chain Reaction- Restricted Fragment Length Polymorphism. J Lab Physicians, 4:1-4.

[11] Allam, A. A. and Salem, I. M. 2012. Evaluation of Rapid Molecular Identification of Clinically Important *Candida Spp* Isolated From Immuno-Compromised Patients Using RF-PCR. J. Am. Sci ,8(2): 463-468.

[12] Yucesoy, M.; Oztek, A. O and Marol, S. 2005. Comparison of three differential media for the presumptive identification of yeasts. Clin Microbiol Infect; 11: 245–247

[13] Pincus, DH.; Orenga, S. and Chatellier, S. 2007. Yeast identification–past, present, and future methods. Med Mycol ; 45:97–121.

[14] Mirhendi, S. H.; Makimura, K.; Khoramizadeh, M. and Yamaguchi, H. 2006: A one- enzyme PCR-RFLP assay for identification of six medically important *Candida* species. Jap. J. Med. Mycol., 47: 225-229.

[15] Shokohi, T.; Hashemi, MB.; Pouri, Z. S.; Hedayati, MT. and Mayahi, S. 2010. Identification of Candida species using PCR-RFLP in cancer patients in Iran. Indian J Med Microbiol., 28: 147-151.

[16] Al-Hashime, H. A. 2000. The Role of *Candida albicans* in Vulvovaginosis. Thesis, M.Sc., Al-Mustansiriya University, the college of science.

[17] Roudbary, M.; Roudbar-mohammadi, S.; Bakhshi, B and Nikoomanesh,F. 2013. Identification of *Candida* species isolated from Iranian women with vaginal candidiasis by PCR-RFLP method. Europ. J. of Exp. Bio., 3(6):365-369.

[18] Zahra, S.; Mahmoudabadi, A. and Majid, Z. 2013. Susceptibility to fluconazole in Candida species Isolated from Vulvovaginal Candidiasis in Ahvaz. Jundishapur J. Microbiol. Special Edition, 2: 8-18.

[19] Habibeh, Z.; Maryam, M.; Roshanak, D.; Allah, N. and Sassan,R. 2013. *Candida* Species in Vulvovaginal Candidiasis Patients from the Town Taibad in Iran; Identified by PCR-RFLP Method. Jundishapur J. Microbiol. Special Edition, 2: 1-6.

[20] Markus, N.; Andreas, G.; Anita, F. and Lell, E. 2002. Impact of N-Chlorotaurine on Viability and Production of Secreted Aspartyl Proteinases of *Candida* spp. Antimicrob. Agents and Chemotherapy, 46: 1996-1999.

[21] Ayatollahi Mousavi, S. A.; Salari, S.; Rezaie, S. and Aghasi, H.2012. Identification of *Candida* Species Isolated From Oral Colonization in Iranian HIV-Positive Patients, by

PCR-RFLP Method. Jundishapur J Microbiol., 5(1):336-340.

[22] Ayatollahi Mousavi, S. A.; Khalesi, E.; Shahidi Bonjar, G.H.;Aghighi,S,; Sharifi, F. and Aram F. 2007: Rapid Molecular Diagnosis for *Candida* species Using PCR-RFLP. Biotechnology, 6: 583- 587.

تشخيص انواع الـ *Candida* المعزولة من مصابات بألتهاب المهبل والفرج بأستخدام تقنية الـ PCR- RFLP

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الخلاصة:

هدفت الدراسة الحالية الى تشخيص انواع الـ *Candida* المسببة لالتهاب المهبل والفرج بأستخدام الطرق المظهرية والجينية كذلك ناقشت الدراسة الحالية تردد أنواع الـ *Candida* بعد عزلها من نساء مصابات . تم جمع 130 عينة 100 من نساء مصابات و30 من نساء سليمات وتم زرعها بأستخدام اوساط زرعية وتم تشخيص الخمائر بأستخدام الطرق المظهرية مثل تكوين انبوب الانبات وانتاج الخيوط الفطرية والسبورات الكلاميدية بأستخدام وسط اكار الذرة المدعم بالتوين 80 واستخدام نظام API20 ووسط كروم جين اكار. كذلك تم تشخيص الخمائر بأستخدام الطرق الجينية ،تقنية الـ PCR-RFLP .وكان تردد الانواع كالاتي *C.albicans* بنسبة تردد 46.4% ثم *C. krusei* وبنسبة 31% و *C. Tropicalis* بنسبة 18% ومن ثم النوع *C. parapsilosis* بنسبة 7.2% واخيرا النوع *C. glabrata* وبنسبة 1.8% . كما ان مناطق ITS1وITS4 تم تضخيمها ومن ثم تم هضمها بأستخدام الانزيم *Msp*1 وبعد الترحيل الكهربائي اعطت كلا من الانواع *C. albicans* وC. *Krusei* وC. *Tropicalis* و *C. glabrata* حزمتين في حين اعطى النوع *C. parapsilosis* حزمة واحده.

الكلمات المفتاحية: تنقية PCR، *Candida albicans*، وسط الكروم، داء المبيضات المهبلي.