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Identification of *Candida albicans* isolated from **Recurrent** Vulvovaginal Candidiasis (RVVC) patients by PCR-RFLP method and its drug sensitivity to *Zataria multiflora* extract

Alireza Farasat[*1], Mohammad sadraeian[2,3], Milad Mohkam[2,3], Shima Akbari Rad[4], Younes Ghasemi[2,3], Mohammad Ali Esfandiary[5], Mosayeb Rostamian[6]

[1]*Young Researchers Club, South Tehran Branch, Islamic Azad University, Tehran, Iran*
[2]*Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[3]*Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran*
[4]*Student Research Committee, Golstan University of Medical Sciences, Gorgan, Iran*
[5]*Reference Quality Control Laboratory of Razak Laboratories Co*
[6] Biotechnology department, Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

Candida species are the most important causes of fungal infections in humans. Vulvovaginal Candidiasis (VVC) covers 20-25 percent of Vulvovaginal forms of infections in women. Candida albicans is the cause of most cases of VVC infection. VVC, especially in the recurrent form, is a physical-mental illness that causes a variety of stresses in patients. Due to side effects of antifungal drugs and increasing drug resistance to these yeasts, researches to find an herbal medicine with fewer side effects and more efficiency should be continued. This study aimed to exactly identify C. albicans species isolated from VVC patients and evaluate its drug sensitivity to Z. multiflora extract. 23 yeasts were Isolated from RVVC patients: 53% C. albicans (15 cases), 35 % C. glabrata (8 cases), and 13 % C. krusei (3 cases). After identification of the yeasts by phenotypic and genotypic methods, cell suspension with 5×10^4 cells of C. albicans was prepared. Drug sensitivity test performed according to Microdilution NCCLS (CLSI) M27-A method in the microplates with various concentrations of Z. multiflora extract. The results showed that the minimum inhibitory concentration (MIC) of the used Z. multiflora extract, for 11, 3, and 1 C. albicans strains, is 8718.75, 17437.5, and 34875 µg/ml, respectively. Our data indicated that this isolated yeasts species from RVVC patients has shown the sensitivity to Z. multiflora extract. It is hoped in the near future that this herbal medicine be the alternative of chemicals drugs and be used to treat RVVC patients.

Keywords: Vulvovaginal Candidiasis, *C. albicans*, *Zataria multiflora*

INTRODUCTION

Candida species are the most important factors for fungal infections in humans and animals. These infections are more common in individuals with background factors. These infections range from mucosal colonization to invasive and fatal infections. Among the different clinical forms of *Candida* infections, cutaneous and mucosal candidiasis are of higher prevalence. Vaginal candidiasis and oral thrush are the most common forms of mucosal candidiasis [1]. Vulvovaginal Candidiasis (VVC) covers 20-25 percent of Vulvovaginal infections [2]. Statistical studies show that prescription of VVC therapy has been doubled since 1980 to 1990 so that the rate has reached to 13 million

prescriptions in 1990 [3]. It is estimated that 75 percent of women experience VVC (at least once) in their lifetime [4]. About 5-8 % of these patients show VVC symptoms, at least four times a year, which this form of the disease is defined as Recurrent Vulvovaginal Candidiasis (RVVC) [5, 6]. This rate has been reported about 40-55 % in some other articles [7]. Due to unknown reasons, the rate of RVVC has been increasing during recent decades [8]. About six million women are suffering from RVVC, annually [9]. *Candida albicans* is responsible for 85-90% of vaginal fungal infections [4]. The rate of non-albicanse *Candida* species in VVC has changed from 9.9 % in 1988 to 17.2 % in 1995 [10]. About 10 -33 % of RVVC cause by non-albicanse species such as *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* [2]. VC, especially in recurrent form, is a physical-mental illness that causes a variety of stresses in patients [10]. It has been proven that the maternal yeast infection during pregnancy may cause thrush in newborns, so it should be seriously tried to eliminate this disease [11]. The use of antifungal therapy has been effective in reducing symptoms in patients, but it is not effective to prevent RVVC [12]. Due to side effects of antifungal drugs and increasing drug resistance to these funguses, researches to find an herbal medicine with fewer side effects and more efficiency should be continued. Due to having phenolic compounds and carvacrol, *Zataria multiflora*, has an antifungal activity on *Candida* isolates [13]. Since past, several methods (each with its own advantages and limitations) have been existed for the identification of yeasts (especially *Candida*. Considering the above mentioned statements and the importance of *Candida* infections, especially VVC as one of the most common opportunistic fungal infections and also high prevalence of *C. albicans* as the cause of VVC, this study aimed to exactly identify the *Candida* species and drug sensitivity of isolated *C. albicans* species from RVVC patients to *Z. multiflora*. It is hoped that our data guide to choice the best treatment, where the etiologic agent have shown resistance to other medicines, and to take a step toward producing a new vaginal gel or ointments [14].

MATERIALS AND METHODS

In this study, 23 isolates of *C. albicans* isolated from RVVC patients were used which these isolates were received from Dr. Shidfar's mycology laboratory (Tehran, Iran) and School of Public Health & Institute of Public Health Research- Tehran University of Medical Sciences.

Morphological Tests:
Morphological Tests including the appearance and color of colonies on CHROMagar *Candida* medium (biomeriux, France), the production of hyphae or pseudohyphae and clamydospores in CMA+TW80 medium (Merck, Germany) and the ability of germ tube production in fresh serum were used to identify the yeast species.

Molecular Tests:
1) DNA extraction
By a bacteriological loop, about 10 mm^3 of a fresh colony was transferred to a 1.5 ml eppendorf tube and then 300 µl of lysis buffer (100 mM Tris pH 8, 10 mM EDTA 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. Then, 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 50 µl distilled water was added and the sample was kept at -20 °C

2) Polymerase Chain Reaction (PCR)
PCR was performed to amplify ITS1-5.8S-ITS2 segment in ribosomal DNA. For this, ITS1-5.8S-ITS2 universal primers were used. The sequences of ITS1 and ITS4 were 5' TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively. The components of PCR reaction were as follows: 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 temperature cycles were as follows: initial denaturation at 95 °C for 6 min, 30 cycles of 30 sec at 94 °C, 45 sec at 56 °C and 1 min at 72 °C and finally 72°C for 7 min [15].

3) Restriction Fragment Length Polymorphism (RFLP):
After confirming PCR products on agarose gel, they were digested by *MSP*1 (Roche Molecular, Germany). The components of digestion reaction were as follows: 10 µl of PCR products, 1.5 µl of digestion buffer, 5 units of *MSP*1 enzyme, and dH2o up to 15 µl. The prepared sample then was placed at 37 °C for 3 hours. Enzymatic digestion of PCR products of different yeasts will produce different patterns. The digested fragments were then electrophoresed through 1.8% agarose gel and then visualized by ethidium bromide staining [15].

The test of anti-candidiasis effects of *Z. multiflora*
In the present work, Microbroth dilution method according to NCCLS $M_{27\text{-}A}$ standard was used [16].

1 - Preparation of Stock solutions of *Z. multiflora* extract
3 ml of pure *Z. multiflora* extract (purchased from Barij Esans, Iran) was dissolved in 5 ml DMSO and used as the stock solution. Concentration of carvacrol and thymol of the purchased extract is 30.6 % w/v and 29.5% w/v, respectively.

2 - Preparation of RPMI-1640 medium
10.4 g of the RPMI-1640 medium powder (51800035 Gibco) and 34.53 g of MOPS buffer [3 - (N-Morpholino) propanesulfonic Acid] were dissolved in one liter of sterile distilled water and pH of the solution was adjusted to 7. Then the solution was filtered using 0.22 µm-pore filters.

Preparation of drug dilution of *Z. multiflora*
In order to prepare dilutions, ten tubes were placed in a rack, then 2 ml RPMI containing MOPS buffer was added to each tube except tube No. 1. Four ml of prepared stock solution was added to tube No. 1. Two milliliter of the first tube (No. 1) was added to the next tube and vortex thoroughly, and then 2 ml of it was added to the next tube, and this process continued until the tube No.10 and 2 ml of the tube No.10 was discarded. Thus, the following dilutions of *Z. multiflora* were achieved.

100 µl of each prepared dilution was added to 48-well sterile cell culture plates with dilution label. Thus ten dilutions: 558 000, 279 000, 139 500, 69 750, 34 875, 17437.5, 8718.75, 4359.3, 2179.6 and1089.8 µg/ml *Z. multiflora* was prepared.

3 - Preparation of inoculums suspension:
The *Candida* species was cultured in Sabouraud Dextrose Agar at 35 °C and was incubated overnight.
1 - 3 similar colonies, which had a diameter of at least 1 mm, were added to 5 ml 0/85% NaCl.
2 - The resulting suspension was thoroughly mixed for 15-20 seconds by vortex.
3 - The number of yeasts was adjusted to 5×10^4 cfu/ml, by making serial dilutions and using Neubauer Chamber,

4 - Inoculation and incubation:
• 0.9 ml of the final inoculum suspension (5×10^4 cfu/ml) was added to each concentration of the extract.
• Three wells were used for positive, negative control, and DMSO.
• Positive control: 0.9 ml of the final yeasts suspension (5×10^4 cfu/ml) + 0.1 ml RPMI
• Negative control: 0.1 ml RPMI
• Control of DMSO: 0.9 ml RPMI + 0.1 ml DMSO
• After mixing, plates were incubated at 37 ° C for 24-48 hours.

For growth control:
10 µl of inoculum suspension and positive and negative was dispensed on Sabouraud Dextrose Agar and incubated plate at 37 ° C for 24-48 hours and the growth rate was controlled.

5 – Reading of medicines MIC results:
Yeasts growth/lack of growth in the wells containing the drug, compared with positive and negative control wells was evaluated using a special mirror and the MIC was recorded. The lowest concentration that prevents growth was considered as the drug MIC of each sample.

In the present study beside isolated strains from patients, *C. albicans* (ATCC: 10261) was used as the control of drug sensitivity testing.

RESULTS

In this study, 23 isolates of *C. albicans* isolated from RVVC patients were used. These isolates were identified and confirmed based on phenotypic diagnostic methods (the color of colonies on CHROMagar *Candida*, lack of germ tube production, and lack of chlamydoconidia production on corn meal agar medium) and genotypic methods. Figure 1 shows the *C. albicans* colonies (green) on chrome agar medium. Figure 2 indicates the fragments derived from enzymatic digestion of the amplified ITS1-5.8S-ITS2 region on the agarose gel.

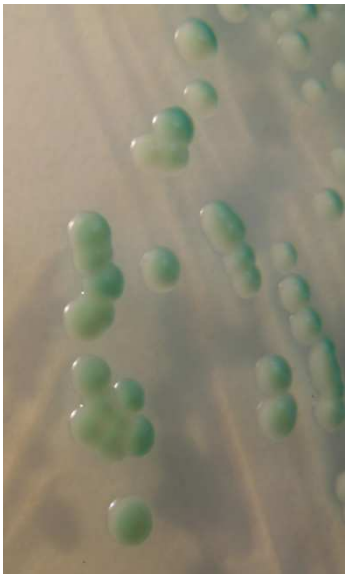

Figure 1: *C. albicans* colonies on CHROMagar *Candida* medium.

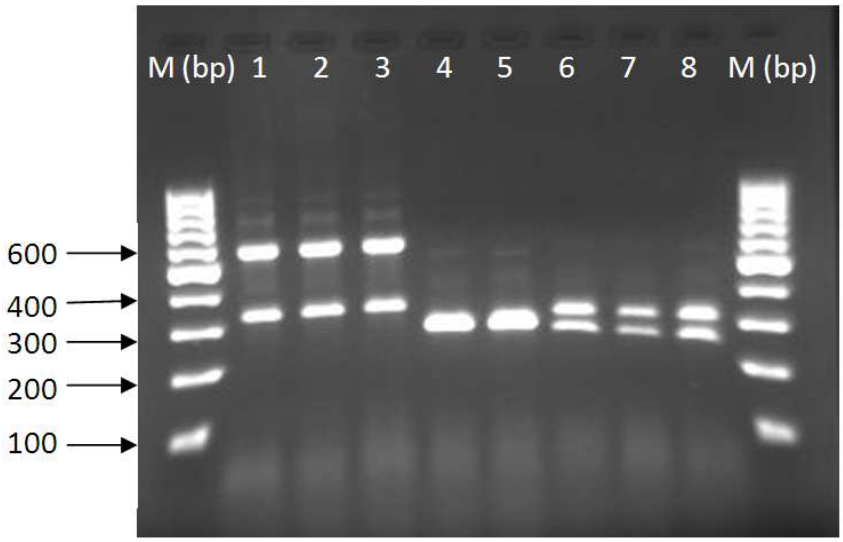

Figure 2: PCR-RFLP profile of *Candida* isolates from RVVC patients, 1, 2, 3: *C. glabrata*, 4, 5: *C. krusei*, 6, 8, 8: *C. albicans*.

Figure 3: Use of a mirror to determine the MIC of *C. albicans strains*.

Table 1: Frequency distribution of the isolates based on MIC of *Z. multiflora extract.*

Species	MIC µ	Number of yeast	percent
C. albicans	8718.75	11	73.4
	17437.5	3	20
	34875	1	6.6
Total		20	100

Results presented in Table 1 show that in dilution, 8718.75 µg / ml of 15 strains of studied *C. albicans* the growth of 11 cases (73.4 %) was inhibited and in this dilution more *C. albicans* has been inhibited.

DISCUSSION

One of the most common candidiasis infections is Vulvovaginal Candidiasis (VVC) which is the second most common form of vaginal infections. Most women, at least once their lives, are faced with this infection that quickly responds to topical antifungal agents. However 5- 8 % of the infected population suffer from repeated attacks and disabling form of the disease which is named "Recurrent Vulvovaginal Candidiasis (RVVC)". More RVVC patients include an active and effective population in society, and therefore the disease would have a direct impact on society. Furthermore the disease forces the patients and community to spend a lot on drugs. It has been reported that *C. albicans* is responsible for 85-90% of vaginal fungal infections [4]. Therefore in this study we tried to evaluate the drug sensitivity of *Candida* species isolated from RVVC patients to *Z. multiflora*. Prevalence of *Candida* species in RVVC patients has been studied several times. In the study conducted by Fidel in 1999, *C. albicans* was the causative agent of 90% of VVC cases and *C. glabrata* was more common among non-albicanse species [17]. In a study in United States which conducted by Richter, 593 cases of vaginal yeasts, including C. *albicans* (420 cases), *C. glabrata* (112 cases), *C. parapsilosis* (30 cases), *C. krusei* (12 cases), *Saccharomycess cerevisiae* (9 cases), *C. tropicalis* (8 cases), *C. lusitaniae* (1 cases) and *Trichosporon* (1 cases) were isolated [18]. In a study conducted in Turkey in 2000 on clinical isolated *Candida* from vagina, the most frequent species were *C. albicans* (50%), *C. glabrata* (26.92 %), *C. krusei* (11.53 %), *C. kefyr* (8.97 %), *C. tropicalis* (1.28 %) and *C. parapsilosis* (1.28 %), respectively [19]. In a study in Belgium in 2002, *C. albicans* was recognized as the most frequent pathogen with a frequency of 68.3 %, followed by *C. glabrata* and *C. parapsilosis* [20]. In another study in the United States in 2005 which conducted by Trama et al., 1316 positive cultures including *C. albicans* (80.2 %), of *C. glabrata* (14.3 %), of *C. parapsilosis* (5.9 %) and *C. tropicalis* (8%) were recognized [21]. According to another study in Bangladesh in 2007 from 172 RVVC patients 125 cases (72.7 %) were *C. albicans*, 29 cases (16.9 %) were *C. glabrata*, 13 cases (7.5 %) were *C. tropicalis*, and about 5 cases (2.9 %) were *C. krusei* [22]. In 2009 a study was conducted on 1050 women, from 215 studied VVC patients in this study, the most frequent species were *C. albicans* (46.9 %), *C. glabrata* (36.7 %), *C. parapsilosis* (10.2 %), *C. tropicalis* (2.8 %), *C. Kefyr* (1.9 %) and *C. krusei* (1.4 %) [23]. Based on a study conducted by Aqhamirian in Iran in 1386 on 128 women suffered from vaginal illnesses, *C.albicans* cover 83 % of isolated fungal species, the 17% remaining cases include *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* [24]. The results of the present study indicated that isolated yeast strains from RVVC patients are shown the sensitivity to *Z. multiflora* extract. It is hoped that in the near future this herbal medicine be an alternative to chemical drugs and used to treat the RVVC patients.

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