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RESEARCH ARTICLE

MOLECULAR DETECTION OF 18S rRNA GENE OF *CANDIDA ALBICANS* ISOLATED FROM PREGNANT WOMEN WITH CLINICAL DIAGNOSIS OF VULVOVAGINITIS

*Saife D. Al-Ahmer

Institute of Genetic Engineering and Biotechnology for Post Graduate Study, Baghdad University, Iraq

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ABSTRACT

Background: Vulvovaginitis is a common disease in women during their lifetime and occurs in diabetes patients, during pregnancy and oral contraceptives users. Although several conventional methods are routinely used for determination; however, vulvovaginitis a challenge for patients and gynecologists.
Aims and objectives: The aims of the present study was to find out the prevalence of candidial vulvovaginitis, along with *Candida albicans* and evolution of the 18S rRNA PCR method sensitivity in the detection of *Candida albicanis* in pregnant women with clinical diagnosis of vulvovaginitis.
Materials and Methods: In this study vaginal swabs from 60 patients were used for Gram stain, culture, Api Candida and PCR analysis. PCR was performed with primer pair targeted to the 18S rRNA gene of *Candida albicans*. The result of the PCR was compared with conventional Gram stain, culture and Api Candida methods. The PCR positive samples were identified by presence of ~ 400 bp amplicon of the internal transcribed spacer (ITS) region of the 18S rRNA gene.
Results: Conventional methods of microscopic examination, candidial culture and Api Candida test gave a positive result in 22 of 60 samples of vulvovaginitis. PCR detected all 22 samples that were positive by conventional method. Three of the 38 samples that were negative by conventional method were positive by PCR. Statistical analysis revealed that the PCR to have a sensitivity of 94.5 % in the detection of *Candida albicans* in vulvovaginitis.
Conclusion: PCR is a sensitive, rapid and useful technique to detect *Candida albicans* in vulvovaginitis.

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INTRODUCTION

Reproductive tract infections, including sexually transmitted infections (STI), are a problem for global public health, especially in developing countries, due to poor health services for prevention, diagnosis, and treatment of these diseases (Pedrosa *et al*., 2011). Besides STI, other conditions that cause imbalance in the normal vaginal flora are emphasized due to their high incidence and symptomatology. *Candida albicans* responsible for infections in the vulva and vagina, causing intense itching and a white, lumpy, and odorless discharge) are among them (Brasil *et al*., 2011). Vaginitis or vaginal candidiasis is a common fungal infection that occurs under predisposing conditions such as, diabetes, antibiotic therapy, oral contraceptive and pregnancy. Disease is a common disorder among women and nearly 75% of women suffer once in their lifetime of genital candidosis (Ehrstrom and Rylander, 2006). In addition, about 5-10% of women suffer from recurrent vaginal candidiasis during their reproductive age (Watson and Calabretto, 2007).

Corresponding author: Saife D. Al-Ahmer,
Institute of Genetic Engineering and Biotechnology for Post Graduate Study, Baghdad University, Iraq.

Vaginitis is not life threatening, however disease may have morbidities in discomfort, pain, and sexual activities. In addition, women with chronic or recurrent vaginitis represent a challenging patient population. Several factors such as stressors (Meyer *et al*., 2006), resistant to antifungal (Novikova *et al*., 2002) and the presence of predisposing factors (Nyirjesy *et al*., 2006) Can cause vulvovaginal relapse in women (Achkar and Fries, 2010; Fidel, 2007). Pregnant women are much affected by adverse effects of these infections, which can have complications including premature birth, premature rupture of membranes, low birth weight, abortion and neonatal death when not diagnosed early and treated properly (Meyers *et al*., 2007; Filip *et al*., 2009; Mendling and Brasch, 2012). During pregnancy, some changes in the lower genital tract, such as hypertrophy of the vaginal walls, increase in both blood flow and temperature, increase in non-specific immunity, and vaginal acidity is typical of this period. Although these changes have a protective function on the uterus, pregnancy and fetus, they may predispose to vaginal infection, requiring special attention in the low-risk prenatal period in order to clarify changes in the vaginal flora and prevent vertical transmission (Alessi *et al*., 2007; Gondo *et al*., 2010). *Candida albicans*

characterized as responsible for 80-95% of vaginal infections (Faro *et al*., 1997).

Therefore, this study aim to use PCR technique as more developed and accurate method to identify *Candida albicans* caused the genital tract infections in pregnant women comparing with conventional methods of *C. albicans* detection.

MATERIALS AND METHODS

Samples collection

Samples were obtained from pregnant women with clinical diagnosis of vulvovaginitis in Al Kadhmiya teaching hospital, Baghdad, Iraq for a period of 3 months. Sixty cases of vulvovaginitis were investigated in this study. Patients' ages ranged from 20 to 35 years of age. This study was carried out after obtaining the approval from the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ Baghdad University and Ministry of Health/ Iraq. A questionnaire was provided based on antenatal care program including name, age, demographic data, symptoms of disease, antenatal visits, pregnancy complications and postnatal data of patients. Vaginal swabs were obtained from 60 patients with clinical diagnosis of vulvovaginitis by gynecologist with a sterile swab. The Gram stains were performed and cultured on Sabouraud's dextrose agar plus chloramphenicol (SC) (Merck, Germany) at 37°C for 48 hours under aerobic condition, then were cultured on chromID Candida agar (Biomerieux, France) at 37°C for 48 hours also under aerobic condition as deferential medium for *Candida albicans* from other *Candida spp*. Api Candida test (Biomerieux, France) was used for diagnosis of the samples as confirm test for *Candida albicans* than other *Candida species.*

DNA extraction

Genomic DNA was extracted from the *Candida albicans* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modification. Briefly, 1 ml of *Candid abicans* culture grown for 20 hours at 37°C in YPD broth (Sigma, USA) was transferred to a 1.5 ml microcentrifuge tube. The samples were centrifuged at 13,000 rpm for 3 minutes to pellet the cells and the supernatant was removed, then the cells were resuspended thoroughly in 300 µl of 50 mM EDTA (Sigma, USA). 20 µl of 20 mg/ml proteinase K (Sigma, Missouri, USA) was added and gently pipet 4 times to mix, then the samples were incubated at 37°C for 30 minutes to digest the cell wall and cooled at room temperature.

The samples were centrifuged at 13,000 rpm for 3 minutes, the supernatant was removed and 300 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added to the cell pellet, then gently pipet to mix. 100 µl of protein precipitation solution (wizard genomic DNA purification kit) was added and vortex vigorously at high speed for 20 seconds, then the samples were sit on ice for 5 minutes and centrifuged at 14,000 rpm for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5 ml microcentrifuge tube containing 800 µl of cold absolute ethanol and gently mixed by inversion until the thread-like strands of DNA form a visible mass, then centrifuged at 14,000 rpm for 10 minutes. The supernatant was carefully decanted and the tubes were drained on clean absorbent paper and 300 µl of room temperature 70% ethanol were added, then the tubes were gently inverted several times to wash the DNA pellet. The samples were centrifuged at 14,000 rpm for 2 minutes and all the ethanol was carefully aspirated. The tubes were drained on clean absorbent paper and the pellet was allowed to air-dry for 10 minutes, then 50 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added. 1.5 µl of RNase solution (wizard genomic DNA purification kit) was added to the purified DNA sample and the sample was vortex for 1 second, then centrifuged briefly in a microcentrifuge for 5 seconds to collect the liquid and incubated at 37°C for 15 minutes. The DNA was rehydrated by incubating at 65°C for 1 hour and the solution was periodically mixed by gently tapping the tube, then the DNA sample was stored at -20°C until use.

DNA quantification

The extracted DNA from the *C. albicans* isolates was quantified spectrophotometrically at OD260/280 nm with ratios 1.5-1.6. The sensitivity of the PFPRIM-F3 and PFPRIM-R4 primers was evaluated by PCR amplification for serial diluted concentrations (10ng-100ng) of purified genomic DNA isolated from *C. albicans.*

Primers selection

The primers for 18S rRNA gene of *Candida albicans* as the target gene for this study were selected according to (Zunaina *et al*., 2008). This set of unique primers was designed based on the conserved region in *Candida albicans*, primers were synthesized by Alpha DNA, Kanda. The primers sequence of 18S rRNA gene and their position of base pair with size of product are shown in (Table 1).

PCR Master Mix

The PCR reactions for detection of 18S rRNA gene of *C. albicans* were performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2.5 µl of 20 pmol PFPRIM-F3 primer and 2.5 µl of 20 pmol PFPRIM-R4 primer and 2 µl of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil.

Table 1. The sequences of primers and their relative binding sites on 18S rRNA gene of *Candida spp.*

Primer name	Sequence 5'-3'	Position of base pair (Candida sp./ Gen-Bank/ accession No. EF120586	Size of product
PFPRIM-F3	GACTCAACACGGGGAAACT	1134-1152	~ 400 bp
PFPRIM-R4	ATTCCTCGTTGAAGAGCA	1509-1526	

PCR program

PCR was carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by (Zunaina *et al*., 2008), with some modification. Briefly, the Amplification of 18S rRNA gene of *C. albicans* was carried out with initial denaturation at 95°C for 6 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 75°C for PFPRIM-F3 and PFPRIM-R4 primers for 90 seconds, and extension at 72°C for 2 minutes. The thermal cycles were terminated by a final extension for 10 minutes at 72°C. Positive control and Nuclease free water as a negative control were used too.

PCR products analysis

The analysis of PCR products of 18S rRNA gene of *C. albicans* were performed on 1% agarose gels. The 100 bp DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The PCR products were stained with ethidium bromide and visualized by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

RESULTS

Conventional methods

Of the 60 pregnant women with clinical diagnosis of vulvovaginitis, Gram stain method yielded positive result of *C. albicans* in 18 patients (30%). Also positive result of *C. albicans* in 16 patients (26.7%) from 60 patients was detected by the culture method using Sabouraud's dextrose agar plus chloramphenicol (SC) (Figure 1) and chromID Candida agar (Figure 2). Whereas Api Candida test show positive result of *C. albicans* in 22 patients (36.7%) from 60 patients (Figure 3).

Figure 1. Culture of *C. albicans* on Sabouraud's dextrose agar plus chloramphenicol (SC) agar

Figure 2. Culture of *C. albicans* on chromID Candida agar

Figure 3. Api Candida test of *C. albicans*

Analysis of extracted DNA of *C. albicans* isolates

After performing of the DNA extraction from *C. albicans* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1% agarose gel at 7volt /cm for 1 hour (Figure 4).

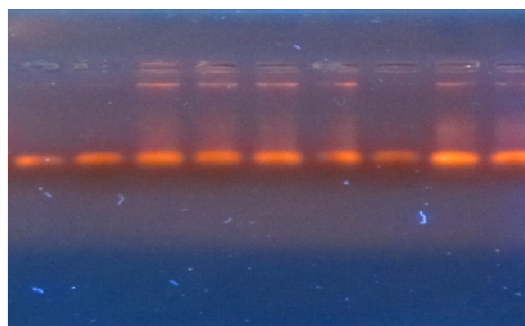

Figure 4. Gel electrophoresis of extracted DNA of *C. albicans* isolates using 1% agarose gel at 7volt /cm for 1 hour. Lane 1-9: Extracted DNA

Analysis of 18S rRNA-based PCR products of *C. albicans* isolates

On the basis of the 18S rRNA sequence region, a product of ~400 bp was amplified by PCR with PFPRIM-F3 and PFPRIM-R4 primers. In the 60 pregnant women with clinical diagnosis of vulvovaginitis, a positive result for *C. albicans* was detected in 25 patients (41.7%) by PCR (Table 2). The PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5 µl of the PCR product were loaded on 1% agarose gel and run at 7volt /cm for 1 hour.

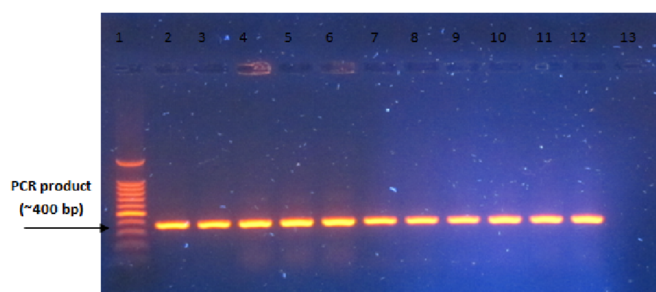

Figure 5. Gel electrophoresis of 18S rRNA-based PCR products of *C. albicans* isolates using 1% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-12: 18S rRNA-based PCR products of *C. albicans* isolates, lane 13: Negative control

The gel was stained with ethidium bromide solution (0.5µg/ml) for 15-30 minutes; finally, bands were visualized on UV transiluminator at 350 wave length and then photographed by using photo documentation system.

Table 2. Laboratory diagnosis of *C. albicans* in pregnant women with clinical diagnosis of vulvovaginitis

Patient No.	Gram stains	Culture	Api Candida test	PCR
1	Oval cell	*Candida albicans*	*Candida albicans*	+
2	GNB	Negative	Negative	-
3	GNB	Negative	Negative	-
4	Oval cell	*Candida albicans*	*Candida albicans*	+
5	GNB	Negative	Negative	-
6	Oval cell	*Candida albicans*	*Candida albicans*	+
7	GNB	Negative	Negative	-
8	GPC	Negative	Negative	-
9	GNB	Negative	Negative	-
10	Oval cell	*Candida albicans*	*Candida albicans*	+
11	NOR	*Candida* sp.	Negative	+
12	GNB	Negative	Negative	-
13	GNB	Negative	Negative	-
14	Oval cell	*Candida albicans*	*Candida albicans*	+
15	GNB	Negative	Negative	-
16	NOR	Negative	Negative	-
17	GPC	Negative	Negative	-
18	GNB	Negative	Negative	-
19	Oval cell	*Candida albicans*	*Candida albicans*	+
20	GNB	Negative	Negative	-
21	Oval cell	*Candida albicans*	*Candida albicans*	+
22	Oval cell	*Candida albicans*	*Candida albicans*	+
23	GNB	Negative	Negative	-
24	Oval cell	*Candida albicans*	*Candida albicans*	+
25	Oval cell	*Candida albicans*	*Candida albicans*	+
26	GPC	Negative	Negative	-
27	Oval cell	*Candida sp.*	*Candida albicans*	+
28	GNB	Negative	Negative	-
29	GPC	Negative	Negative	-
30	NOR	*Candida sp*	*Candida albicans*	+
31	GNB	Negative	Negative	-
32	Oval cell	*Candida albicans*	*Candida albicans*	+
33	NOR	*Candida sp.*	*Candida albicans*	+
34	NOR	*Candida* sp.	Negative	+
35	GPC	Negative	Negative	-
36	GPC	Negative	Negative	-
37	GNB	Negative	Negative	-
38	NOR	*Candida sp.*	*Candida albicans*	+
39	GNB	Negative	Negative	-
40	Oval cell	*Candida albicans*	*Candida albicans*	+
41	NOR	Negative	Negative	-
42	GPC	Negative	Negative	-
43	GNB	Negative	Negative	-
44	GPC	Negative	Negative	-
45	Oval cell	*Candida sp.*	*Candida albicans*	+
46	GNB	Negative	Negative	-
47	Oval cell	*Candida albicans*	*Candida albicans*	+
48	GNB	Negative	Negative	-
49	GNB	Negative	Negative	-
50	Oval cell	*Candida albicans*	*Candida albicans*	+
51	GNB	Negative	Negative	-
52	NOR	Negative	Negative	-
53	GNB	Negative	Negative	-
54	Oval cell	*Candida albicans*	*Candida albicans*	+
55	GNB	Negative	Negative	-
56	NOR	*Candida* sp.	Negative	+
57	GPC	Negative	Negative	-
58	GNB	Negative	Negative	-
59	NOR	*Candida sp.*	*Candida albicans*	+
60	Oval cell	*Candida albicans*	*Candida albicans*	+

PCR result was considered positive for *C. albicans* when there was presence of ~400 bp PCR product band on agarose gel electrophoresis. No amplification was observed with negative control (Figure 5).

DISCUSSION

In this study, the most common initial laboratory procedure done in diagnosis of candidial vulvovaginitis is Gram stains method.

In this study, a positive Gram stains were observed in 30% (18) of the patients. The percentage of positive stains showing fungal elements was similar to studies in South Florida, in which 33% of Gram stains were positive (Rosa and Alfonso, 1994). Culture yielded positive results in 26.7% (Zunaina *et al*., 2008) of the patients and Api Candida test show positive results in 36.7% (22) of the patients whereas the PCR was positive in 41.7% (25) of the patients. The findings of this study agree with those of Lohmann *et al.* (1998) and Anand

et al. (2001), demonstrating high analytical specificity and sensitivity of the PCR method compared with the conventional method. Conventionally, culture has been used to detect candidial pathogens, but takes two days to one week for final identification. The main problem with culture from vaginal swabs is the small amounts of vaginal secretions that can be obtained for diagnosis, increasing the negative results risk of candidial culture also some *Candida* isolates might be grow slowly. This may explain the negative culture results in 3 of the samples used in this study. In current study, 18S rRNA specific PFPRIM-F3 and PFPRIM-R4 primers that amplify medically important *C. albicans* were selected and used. These primers are based on the conserved region of 18S rRNA gene which is designed to detect wide range of fungal strains with the PCR product size of ~400 bp (Ferrer *et al*., 2001).

The 18S rRNA gene is a multi-copy gene that is slowly evolving and highly conserved among fungi, making it an attractive target for the detection of *Candida* yeast in clinical specimens. Detection of candidial etiology by 18S rRNA targeted PCR will be useful in early diagnosis of candidial vulvovaginitis and could help in early initiation of anticandidial therapy (Faro *et al*., 1997). This study demonstrates that 18S rRNA-based PCR has high degree of sensitivity for the detection of medical significant *Candida albicans*. The PCR diagnostic test had a sensitivity of 94.5%. PCR detected all of the 22 samples that were positive by conventional method. One of the samples that were negative by PCR is a case of microbial vaginitis. This could be due to insufficient candidial elements present in vaginal swab or due to sequence variation of 18S rRNA gene of this candidial isolate. Chen *et al.,* in 2000 have used ITS2 region specific primers to detect fungal species based on PCR product size (ranging from 237 to 429 bp) identification by capillary electrophoresis and restriction polymorphism (Latha *et al*., 2014). In most cases of vaginitis, the most important laboratory information that the gynecologist needs to know is whether the infectious agent is fungal (candidial) or bacterial. They often hesitate to initiate anticandidial therapy in candidial culture negative cases due to the risk of drug associated toxicity. Positive PCR results that are available earlier than culture will justify the use of anticandidial agents promptly, resulting in improved visual outcome. This data agrees with the study by Anand *et al.* (Chen *et al*., 2000) and confirms the efficacy of the PCR assay compared to conventional methods of diagnosis in the clinical setting.

Conclusion

This study has demonstrated the efficacy of the 18S rRNA gene to detect *C. albicans* in clinical samples. This PCR based test is a rapid, sensitive and a useful method to detect cadidial etiology in the pregnant women with clinical diagnosis of vulvovaginitis when compared with conventional laboratory diagnosis of *C. albicans*. Further studies with larger sample size are needed to refine the technique, to calculate sensitivity and specificity, and to establish the value of the technique in managing pregnant women with vaginal candidiasis.

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