Molecular typing and \textit{in vitro} fluconazole susceptibility of \textit{Candida} species isolated from diabetic and nondiabetic women with vulvovaginal candidiasis in India

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\textbf{KEYWORDS}
C \textit{Glabrata}; Diabetes; India; Molecular typing; Vulvovaginal candidiasis

\textbf{Background:} \textit{Candida albicans} and \textit{Candida glabrata} are the major causes of vulvovaginal candidiasis (VVC) in Indian women with diabetes mellitus. Little information is available regarding the genotyping of \textit{Candida} species isolated from Indian diabetic women with VVC.

\textbf{Methods:} In this study, a total of 57 \textit{Candida} species, comprising \textit{Candida albicans} and \textit{Candida glabrata}, isolated from Indian women with VVC, were genotyped and tested for fluconazole susceptibility. Arbitrarily primed polymerase chain reaction (AP-PCR) was used to genotype \textit{C glabrata} isolates, whereas Southern blot hybridization using a \textit{Candida albicans} repetitive element-2 (CARE-2) probe was used to genotype \textit{C albicans}.

\textbf{Results:} Genotyping showed that all the \textit{C albicans} isolates were genetically heterogenous. The pattern of DNA bands obtained after AP-PCR for \textit{C glabrata} strains were predominantly conformed to genotype A. \textit{In vitro} fluconazole-susceptibility testing of the isolates using the Clinical and Laboratory Standards Institute M27A2 protocol showed that more than 93\% of the \textit{Candida} isolates were susceptible.

\textbf{Conclusions:} Ninety-five percent of the \textit{C albicans} isolates analyzed were different and genetically unrelated. The analysis of the AP-PCR DNA banding pattern of \textit{C glabrata} isolates showed that it resembled genotype “A”. The \textit{Candida} isolates were found to be susceptible to fluconazole, with minimum inhibitory concentrations ranging from 0.5 \(\mu\)g/mL to 8 \(\mu\)g/mL. This correlates with the use of fluconazole as a first-choice antifungal for treating VVC in India.

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Introduction

Patients with diabetes mellitus (DM) are at risk of vulvovaginal candidiasis (VVC).1,2 Candida albicans is still one of the most common species isolated in women with VVC. Recent studies have shown that non-albicans species of Candida are the major cause of such infections in patients with DM compared with those in nondiabetic individuals.1,3 Candida glabrata is the most common non-albicans Candida species isolated from women with DM and VVC.1,3 We hypothesized that the differences in the prevalences of C glabrata infection in patients with or without DM could be the result of the differences in the molecular genotypes of species from these groups of patients with or without DM.

Recently, Becker et al.4,5 characterized C glabrata strains isolated from nondiabetic women by arbitrarily primed polymerase chain reaction (AP-PCR) with multiple sets of primers. Three distinct molecular subtypes were identified by primer AP50-1.4,5 Candida albicans—specific DNA probe "CARE-2" has also been used recently for genotyping Candida isolates.6−8 The present pilot study was, therefore, conducted to assess the differences in the genotypes between C albicans and C glabrata isolates recovered from diabetic women with VVC attending a tertiary care hospital in New Delhi, India. The minimum inhibitory concentrations (MICs) of fluconazole against all the isolates were also determined.

Methods

Test isolates

A total of 57 isolates of Candida species were evaluated. Out of these, 22 and 7 were C glabrata obtained from diabetic and nondiabetic women, respectively. Also included were 21 and 7 C albicans isolated from diabetic and nondiabetic women, respectively. These isolates were identified and characterized previously by Goswami et al.1,3 The isolates were preserved in 15% glycerol and stored at −80°C.

All of the isolates included in this study were collected randomly from diabetic and nondiabetic women with VVC symptoms. All of them were attending the clinic as outpatients of the All India Institute of Medical Sciences, New Delhi, India. Samples were collected with two sterile cotton swabs to collect discharge from high vagina.1,3 One swab was used to detect the presence of any yeast by Gram staining, and the other was used to test growth on Sabouraud dextrose agar slants containing 2 μg of gentamicin per mL and 0.5% cycloheximide. In the case of positive growth, yeast identification was done by conventional methods. The method used was the germ tube production test, which was done by following the isolation of a single colony into 0.5 mL of horse serum, followed by incubation at 37°C for 2 hours. Morphology testing for the presence of chlamydospores, pseudohyphae, true mycelium, and blastospore arrangement was done on corn meal agar. An enzymatic triphenyl tetrazolium chloride reduction test was performed, in which each Candida species grows with a distinct texture and color. For further characterization, each isolate was subjected to carbohydrate assimilation and fermentation tests.1,3,6

DNA extraction

Chromosomal DNA was isolated from each sample using conventional technique after lysis of the cells by Zymolyase (Seikagaku Biobusiness Corporation, Japan) treatment as described previously.6

Genotyping of Candida glabrata

Genotyping of C glabrata was done by AP-PCR. AP50-1 primer (5'-GATTCCAGACC-3') was used to amplify chromosomal DNA in 50 μL of PCR reaction mixture as described previously by Becker et al.4,5 Briefly, the PCR mixture contained Deoxy-nucleotides Triphosphates (dNTPs) 100 μM (each) dATP, dCTP, dGTP, and dTTP; enzyme buffer; 50 pmol primer; and 2.5 U Taq-polymerase. The amplification was performed in an automated thermocycler (iCycler, Bio-Rad, Philadelphia, PA, USA). The thermal cycling conditions were 30 cycles of denaturation at 94°C for 1 minute (2 minutes for the first cycle), annealing at 35°C, and extension at 72°C for 2 minutes. Amplified products (50 μL) were resolved by 1% agarose gel electrophoresis at 100 V for 1.5 hours. The gel was stained with ethidium bromide; exposed to UV light to visualize the amplified products; and photographed (Multimage, Alpha Innotech Corporation, Santa Clara, CA, USA). All experiments were performed in triplicates. Because of low discriminatory power of AP-PCR, caution should be practiced in the interpretation of AP-PCR data.

Genotyping of Candida albicans

DNA fingerprinting of the C albicans isolates by Southern blot hybridization was done as described earlier.6 In brief, chromosomal DNA (2 μg) from each isolate was digested with restriction enzyme EcoRI till completion at 37°C for 2 hours. The digested DNA samples were then electrophoresed on agarose gel (0.8%) in 1× Tris/Borate/EDTA (TBE) buffer (89 mM Tris-borate and 1 mM EDTA) by applying a voltage gradient of 2 V/cm for a period of 20 hours; stained with ethidium bromide (0.5 μg/mL); destained; visualized under UV; and photographed using gel documentation system (Multimage). Separated DNA fragments were denatured in situ using an alkali and were then neutralized with an acid. The denatured DNA fragments were then transferred to nylon membrane (Sigma-Aldrich, St. Louis, MO, USA) by capillary action. The transferred DNA fragments were then UV cross-linked (Stratagene, Santa Clara, CA, USA) to the membrane and prehybridized in 300 mM phosphate buffer containing 7% sodium dodecyl sulfate (SDS) and 1 mM EDTA at 65°C for 2–4 hours. Immobilized DNA fragments were hybridized with C albicans—specific probe CARE-2 labeled with [α-32P]dATP (Amersham Pharmacia Biotech, Buckinghamshire, UK) at the same temperature for 16 hours.6 Briefly, CARE-2 probe was prepared as follows.

Labeling of CARE-2 probe

About 5−10 μg of plasmid DNA (pRFL37) containing the CARE-2 sequence was digested with restriction enzyme KpnI and PstI, and the generated DNA fragments were separated
on 1% agarose gel ran in 1 × TBE buffer. The 1.06-kb DNA fragment containing the CARE-2 sequence was then cut out of the gel, homogenized, and suspended in 1 × Tris-EDTA (TE) buffer and eluted out of the agarose. The purified DNA fragment was hybridized to random primers (Takara, Shiga, Japan) and extended in presence of 200 μM each of dGTP, dCTP, and dTTP, and 10 μCi [α-32P]dATP (6,000 Ci/mmol), by Klenow fragment at 30°C for 30 minutes; the labeled DNA fragment was then precipitated with absolute ethanol and washed several times with 70% ethanol to get rid of unincorporated nucleotides. Finally, the precipitated DNA was dissolved in hybridization buffer and used for hybridization.

After hybridization, the nylon membrane was washed several times with 2× sodium chloride and sodium citrate, containing 0.1% SDS; exposed to X-ray film at −80°C for 16–24 hours; and developed. All experiments were carried out in triplicate. The DNA fingerprinting was further confirmed when analyzed quantitatively using Dendron software package, version 3.0 (Solltech, Iowa City, IA, USA) as described by Pujol et al.9

**In vitro tests for fluconazole susceptibility**

Susceptibility testing of isolates to fluconazole was carried out as stipulated in the National Committee for Clinical Laboratory Standard CLSI/NCCCLS M27A2 protocol (2002). Briefly, fluconazole (Ranbaxy, Haryana, India) was dissolved in sterile distilled water. The drug was serially diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) with L-glutamine but without sodium bicarbonate and buffered at pH 7.0 with morpholinepropanesulfonic acid. The final drug dilution ranged from 0.125 μg/mL to 64 μg/mL. The inocula were prepared in 0.9% sterile normal saline from five to seven single *Candida* colonies grown on Sabouraud’s dextrose agar for 16 hours at 37°C to attain a turbidity equivalent to 0.5 MacFarland Standard.

Microdilution plates were set up, and each test plate had two drug-free-growth controls, one with media alone (sterility control) and the other with media and test isolate *Candida* isolates without fluconazole (growth control). Quality control was performed by testing CLSI-recommended strains *Candida krusei* ATCC 6285 and *Candida parapsilosis* ATCC 22019. The plates were incubated at 35°C and read after 48 hours of incubation. Endpoint determinations were made by visually comparing the growth in the wells containing the drug with the growth in the drug-free wells. The MIC reported at 80% (MIC80) was the lowest concentration of fluconazole that caused 80% inhibition of growth.

**Results**

Figure 1 shows the genotyping pattern observed after AP-PCR of *C. glabrata* isolates obtained from 22 patients with VVC and DM, and seven patients without DM but with VVC. The AP50-1 primer used showed good band pattern with high percentage of polymorphism. All *C. glabrata* strains tested showed the specific amplification products to the primer used. One main cluster profile was observed for all the isolates of *C. glabrata* (Figs. 1A and 1B) from diabetic and nondiabetic patients. Three distinct major bands characterized the first cluster designated as “A.”4,5 The DNA banding patterns obtained in all of them were analyzed and were found to be similar to genotype “A.”

The DNA fingerprinting patterns of the 21 isolates of *C. albicans* from the patients with diabetes and eight isolates from the nondiabetic individuals are shown in Figs. 2A and 2B. The CARE-hybridization patterns of these isolates were found to be relatively complex (Fig. 2). The genotyping pattern of each of the isolates was specific for individual *Candida* isolates and did not relate to one another both in the diabetic and in the nondiabetic groups. This further confirmed that all the *C. albicans* isolates were different and genetically unrelated, except Isolates 1 versus 5 from diabetic women and Isolates 5 versus 6 from nondiabetic women. The DNA genotyping pattern was further confirmed by Dendron software package, version 3.0 (Fig. 3). Our data showed unrelatedness of most of the *C. albicans* isolates, which may indicate different sources of infection.

Discussion

There are relatively few studies that highlight the problem of VVC among Indian diabetic patients; thus, it remains a poorly investigated area. In recent years, VVC caused by *C. glabrata* has been increasingly isolated from women with DM. First, we analyzed the genotyping of *C. glabrata* isolates to get an insight on the genotypes of *C. glabrata* that cause VVC in Indian women. There is a wide prevalence of *Candida* species in patients with DM.1,10 In a previous study by Goswami et al.,1 it was found that the occurrence of VVC was significantly (*p* = 0.0025) higher in diabetic women (46%) compared with that in nondiabetic women (23%). In a study by Nowakowska et al.10 a similar but increased prevalence was observed among diabetic pregnant women (40.4%) compared with that in nondiabetic pregnant women (13.6%). The increased prevalence of *Candida* species infection among diabetics has long been postulated to be either because of host- or pathogen-related factors.11 Most of the pathogens, including *Candida* species, have developed an effective battery of putative virulence factors and particular strategies that help them to colonize host tissues, cause disease, and overcome host defenses.12–14 The site of infection (host factors) has a direct correlation with the virulence of the *Candida* species. The vulvovaginal tissues have unique conditions that may induce the virulence of the *Candida* species, which include the pH, the temperature, the adherence capacity expression of *Candida*, and the concentrations of the nutritional material that are necessary for the virulence of *Candida* species.12–14
In the present study, the DNA banding pattern of *C. glabrata* isolates might belong to the "A" genotype. Specific and individualized genotype in all the cases indicated endogenous rather than exogenous source of infection (from the environment, contamination while examining the patients, or while performing mycological tests in the laboratory). Different genotypes of all the isolates and lack of differences between diabetic and control groups also

![Figure 1](image1.png)

(A) Agarose gel patterns demonstrating the AP-PCR with AP50-1 primer of the representative *Candida glabrata* isolates obtained from diabetic patients with VVC. (B) Agarose gel patterns demonstrating the AP-PCR with AP50-1 primer of the *C. glabrata* isolates obtained from nondiabetic patients with VVC (total 7 isolates). The numbers on the top of the figure indicate isolate numbers. The arrows point out the main band patterns. AP-PCR = arbitrarily primed polymerase chain reaction; M = molecular weight marker (1-kb ladder; New England Biolabs, Ipswich, MA, USA); VVC = vulvovaginal candidiasis.

![Figure 2](image2.png)

(A) DNA fingerprinting patterns of *Candida albicans* isolates (21 isolates) obtained from diabetic patients with VVC—EcoR1-digested genomic DNA—were hybridized with labeled *C. albicans*—specific CARE-2 probe. Lanes 7, 8, 14, 22, and 23 represent *C. glabrata* isolates (negative control, no hybridization bands with CARE-2 probe); the rest are all *C. albicans* isolates. (B) DNA fingerprinting patterns of *C. albicans* isolates obtained from nondiabetic patients with VVC—EcoR1-digested genomic DNA—were hybridized with labeled CARE-2 probe. Lanes 8, 9, 10, 11, and 12 represent *C. glabrata* isolates (negative control, no hybridization bands with CARE-2 probe); the rest are all *C. albicans* isolates. The numbers on the top of the figure indicate isolate numbers. M = molecular weight marker (1-kb ladder; New England Biolabs); C = control *C. albicans* strain (ATCC 10261); VVC = vulvovaginal candidiasis.
This is consistent with our previous study on oropharyngeal candidiasis in human immunodeficiency virus/AIDS patients.6

In vitro fluconazole-susceptibility testing of all the isolates showed that more than 93% of the Candida isolates were susceptible. Factors contributing to fluconazole resistance are numerous and include the degree of immunosuppression and prior fluconazole exposure of the patient, the contribution of other chemotherapeutic drugs, and the intrinsic resistance of Candida species. In the past, antifungal drug prophylaxis was rarely practiced in India. However, over the years, the situation is gradually changing and increasing number of Indian patients are now receiving fluconazole therapy, which is the most widely used drug for treating VVC in India.1–3 In a comparison of our in vitro drug susceptibility data with those of Houang et al.’s17 clinical study, where they reported that the oral therapy of administration of a single dose of 150 mg of fluconazole for nondiabetic women with VVC inhibited the growth of 90% of Candida species isolates, a clinical correlation between the in vitro fluconazole-susceptibility profile and clinical response was found.

This study is probably the first genotyping VVC study of diabetic women in India in which comprehensive data were generated; this will help not only in patient management but also in future clinical research. More studies using greater number of samples and repeated collection of isolates from the same patients at different time intervals are warranted.

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**References**
