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ORIGINAL ARTICLE

Molecular typing and *in vitro* fluconazole susceptibility of *Candida* species isolated from diabetic and nondiabetic women with vulvovaginal candidiasis in India

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Background: Candida albicans* and *Candida glabrata* are the major causes of vulvovaginal candidiasis (VVC) in Indian women with diabetes mellitus. Little information is available regarding the genotyping of *Candida* species isolated from Indian diabetic women with VVC.
Methods: In this study, a total of 57 *Candida* species, comprising *Candida albicans* and *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 *C glabrata* isolates, whereas Southern blot hybridization using a *Candida albicans repetitive* element-2 (CARE-2) probe was used to genotype *C albicans*.
Results: Genotyping showed that all the *C albicans* isolates were genetically heterogenous. The pattern of DNA bands obtained after AP-PCR for *C glabrata* strains were predominantly conformed to genotype A. *In vitro* fluconazole-susceptibility testing of the isolates using the Clinical and Laboratory Standards Institute M27A2 protocol showed that more than 93% of the *Candida* isolates were susceptible.
Conclusions: Ninety-five percent of the *C albicans* isolates analyzed were different and genetically unrelated. The analysis of the AP-PCR DNA banding pattern of *C glabrata* isolates showed that it resembled genotype "A". The *Candida* isolates were found to be susceptible to fluconazole, with minimum inhibitory concentrations ranging from 0.5 μg/mL to 8 μg/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 the inoculation 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'-GATTCAGACC-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 Deoxynucleotides Triphospahte (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 (MultiImage, 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 *Eco*RI 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 (MultiImage). 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-^{32}P]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 *Kpn*I and *Pst*I, 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/NCCLS 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-Ardrich) with L-glutamine but without sodium bicarbonate and buffered at pH 7.0 with morpholinepropanesulfonic acid. The final drug dilution ranged from 0.125 ug/mL to 64 ug/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 krusie ATCC 6285 and Candida parapsilsosis 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.

The comparison of in vitro fluconazole-susceptibility patterns in diabetic and nondiabetic women revealed that more than 93% of the Candida isolates obtained from diabetic women were susceptible, with MICs ranging from 0.5 μg/mL to 8 μg/mL, and the most common MIC among diabetic women (mode) was 4 μg/mL. Two of the Candida isolates (C albicans Isolate 21 and C glabrata Isolate 10) tested were found to be dose-dependently susceptible, with MICs ranging from 16 μg/mL to 32 μg/mL. However, C glabrata Isolate 7 and two C albicans isolates (Isolates 9 and 15) were found to be resistant, with MICs greater than or equal to 64 μg/mL. All the Candida isolates obtained from nondiabetic women were fluconazole susceptible (0.5−8 μg/mL) and mode of 2 μg/mL.

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]
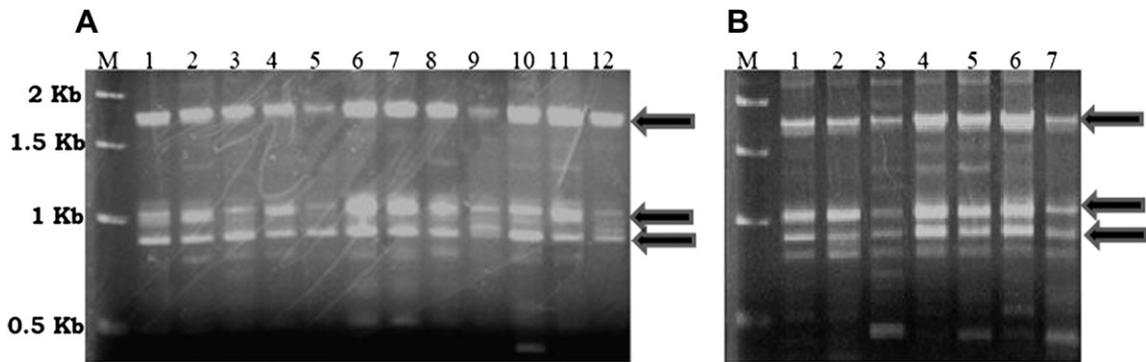
Figure 1. (A) Agarose gel patterns demonstrating the AP-PCR with AP50-1 primer of the representative *Candida glabrata* isolates obtained from diabetic patients with VVC. (B) Agarose gel patterns demonstrating the AP-PCR with AP50-1 primer of the *C glabrata* isolates obtained from nondiabetic patients 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.

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
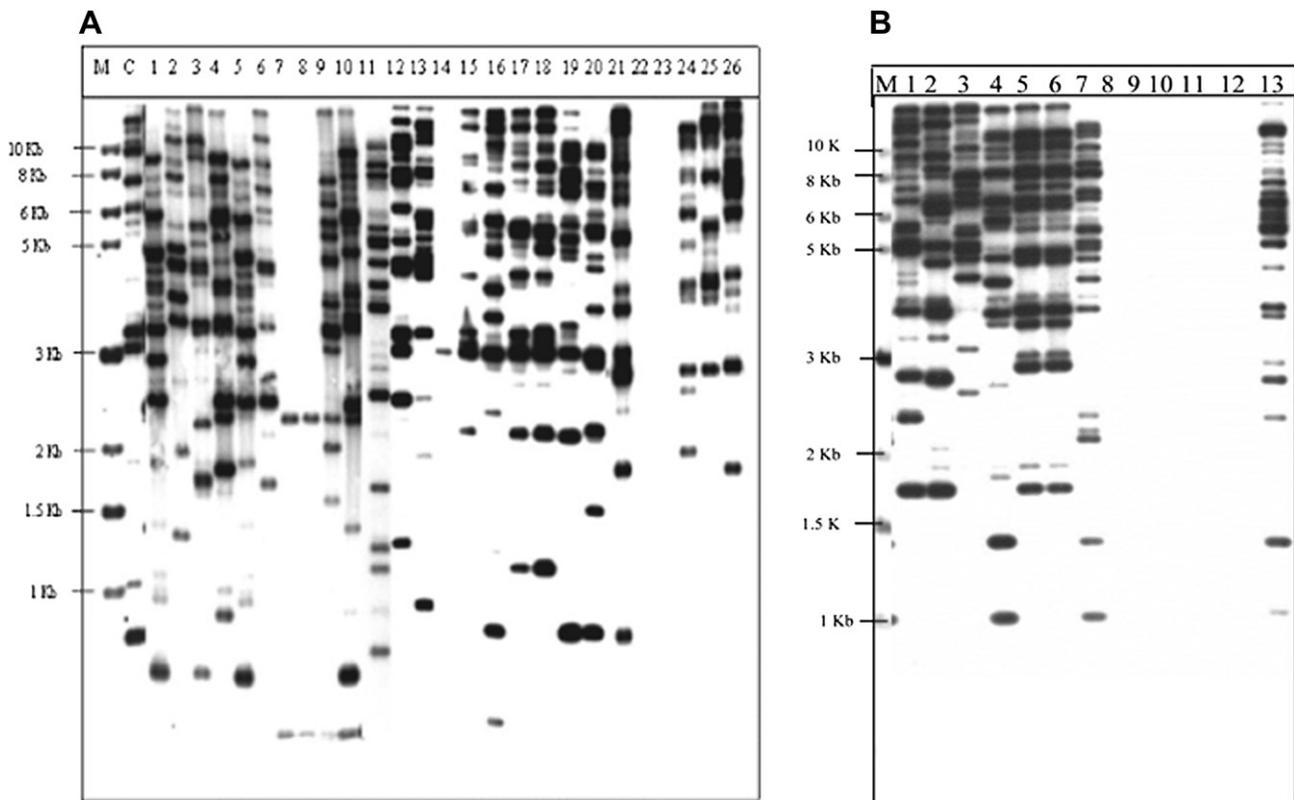
Figure 2. (A) DNA fingerprinting patterns of *Candida albicans* isolates (21 isolates) obtained from diabetic patients with VVC—*Eco*R1-digested genomic DNA—were hybridized with labeled *C albicans*—specific CARE-2 probe. Lanes 7, 8, 14, 22, and 23 represent *C glabrata* isolates (negative control, no 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—*Eco*R1-digested genomic DNA—were hybridized with labeled CARE-2 probe. Lanes 8, 9, 10, 11, and 12 represent *C glabrate* 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.

Diabetic dendro
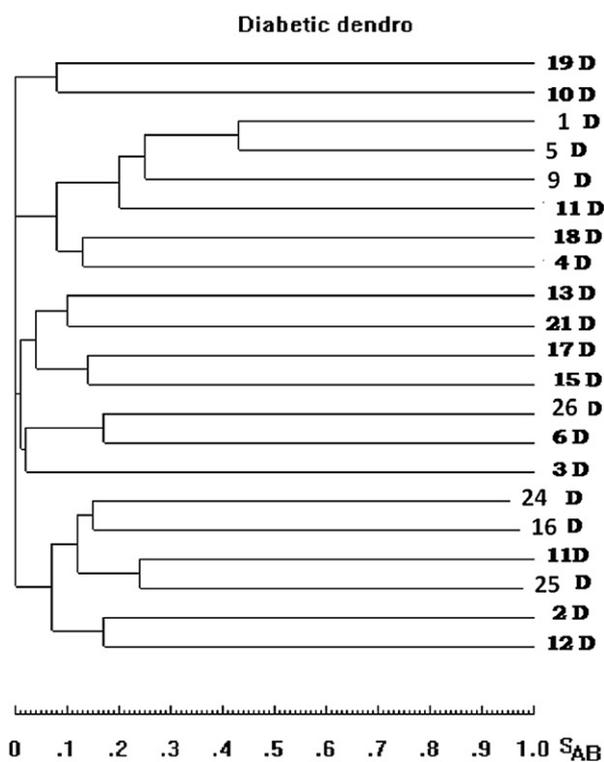
Figure 3. Dendrogram generated from the similarity coefficient (S_{AB}s) compared all possible pairs of the 21 isolates fingerprinted with the CARE-2 probe.

indicate that host-related factors are predominantly involved in the pathogenesis of the widely prevalent *Candida* infection among patients with DM. In a study by Lian et al.[15] on VVC in nondiabetic women, it was concluded that the AP-PCR amplification products were similar among isolates from different individuals. In India, there appears to be a higher glycosylated hemoglobin (HbA1) level in patients with DM and VVC as compared with diabetics without VVC ($12.8 \pm 2.6\%$ *vs.* $9.7 \pm 1.7\%$, respectively; $p = 0.001$).[1] Nowakowska et al.[10] have also highlighted the predominant role of poor glycemic control in the high prevalence of VVC in pregnant women with DM. Lack of genetic differences in the *Candida* species between diabetic group and control group indicates that much emphasis should be placed on achieving better glycemic control in diabetic patients so as to decrease the rate of *Candida* prevalence in such infections in them.

The pattern of DNA genotyping was specific for each *C albicans* isolate and did not correlate with each other in the diabetic and nondiabetic groups. The heterogenous nature of the DNA fingerprinting was further confirmed when analyzed quantitatively using Dendron software package, version 3.0, as described by Pujol et al.[9,16] This indicates that most of the *C albicans* isolates were different and genetically unrelated (Fig. 3). This seems to suggest that there were different sources of *Candida* infection among the patients sampled. Although candidiasis is mainly an endogenous infection, it is not possible to speculate the primary source of infection. However, we cannot rule out the possibility that a commensal may suddenly become a pathogen if the existing conditions permit. 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.'s[17] 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.

Acknowledgments

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