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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