Original Article

Antifungal susceptibility and molecular typing of 115 Candida albicans isolates obtained from vulvovaginal candidiasis patients in 3 Shanghai maternity hospitals

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Abstract

In our multicenter study, we studied the distribution of Candida species in vulvovaginal candidiasis patients and investigated antifungal susceptibility profile and genotype of Candida albicans in vaginal swab. A total of 115 Candida albicans strains were detected in 135 clinical isolates. Minimum inhibitory concentration determinations showed that 83% and 81% of the 115 Candida albicans strains were susceptible to fluconazole and voriconazole. Randomly amplified polymorphic DNA analysis (RAPD) was applied to identify clonally related isolates from different patients at the local level. All tested strains were classified into genotype A (77.4%), genotype B (18.3%), and genotype C (4.3%). Genotype A was further classified into five subtypes and genotype B into two subtypes. Candida albicans was the dominant pathogen of vulvovaginal candidiasis, the majority belonging to genotype A in this study. Exposure to azoles is a risk factor for the emergence of azole resistance among Candida albicans isolated from VVC patients.

Key words: Candida albicans, random amplified polymorphic DNA, gene homology.

Introduction

Vulvovaginal candidiasis (VVC) is a common illness attributed to an overgrowth of Candida species, and it has been estimated that 75% of all women will experience an episode of VVC during their lifetime. VVC is the second most common cause of vaginitis after bacterial vaginosis with Candida albicans accounting for between 80–95% of all episodes of VVC worldwide1,2. It is noteworthy that recurrent VVC affects about 5% of all women of childbearing age3.
More than 20 Candida species have been identified as human pathogens\textsuperscript{4-7}. Research has shown that in majority countries, the most common species detected in patients with VVC were *Candida albicans* (65–90%), non-albicans species, particularly *Candida glabrata*, and in rare cases *Saccharomyces cerevisiae*, which causes less than 10% of all cases of vulvovaginitis, although there was some regional variation. The latter was generally associated with milder clinical symptoms compared to those that present in *Candida albicans*-associated vaginitis\textsuperscript{8-11}.

There is only limited information regarding the antifungal susceptibility and molecular epidemiology of *Candida albicans* isolates in patients suffering from vulvovaginal candidiasis in China. Despite the small number of samples analyzed, this study contributes to the understanding of the distribution of Candida species in vulvovaginal candidiasis patients and the susceptibility of *Candida albicans* to the most commonly prescribed systemic antifungal drugs and the molecular relationship and genetic diversity among 115 clinical *Candida albicans* isolates obtained from patients in three maternity hospitals in the Shanghai region of China. Our data also provides experimental evidence for appropriate clinical monitoring and the correct therapeutic strategy to treat vulvovaginal candidiasis.

**Methods**

**Identification of strains**

The present retrospective study collected 135 patients from the Obstetrics and Gynecology Hospital of Fudan University, International Peace Maternity and Child Health Hospital and Shanghai First Maternity and Infant Hospital, between November 2013 and January 2014. These patients presented with VVC and all had a history of at least one prior episode of VVC, occurring from several months to several years prior to this visit. All of these patients had symptoms consistent with VVC, which included vaginal itching, vulval soreness and irritation, pain or discomfort during sexual intercourse, pain or discomfort during urination, white and thick vaginal discharge, pain with sex, and redness around the vagina. The most common symptom in this population was vaginal itching, and some patients had more than one symptom. These patients had all received antifungal treatment for prior VVC. Some patients received fluconazole 150 mg, oral for 7 days. Some received miconazole suppository (200 mg, 7 days), or clotrimazole suppository (150 mg, 7 days). None of the patients visited the clinic routinely. No patients were positive for human immunodeficiency virus. Approval for the research was obtained from the Research Ethics Committee of the three maternity hospitals and written consent was obtained from all patients participated in the study.

All samples were collected by vaginal swab, and only one yeast organism was isolated from each vaginal swab. Swabs were cultured on CHROMagar Candida (Chromagar, Paris, France) and Yeast Extract Peptone Dextrose Medium (ShengGong, Shanghái, China). All isolates were identified by API 20C AUX (Biomérieux, Lyon, France)\textsuperscript{12}. We collected one isolate per patient.

**Antifungal susceptibility testing**

Antifungal susceptibility testing for fluconazole and voriconazole was determined by the broth microdilution method using ATB FUNGUS 3 (Biomérieux, France), according to the guidelines outlined in CLSI document M27-S\textsuperscript{4}\textsuperscript{13-14}. An ATB FUNGUS 3 strip consists of 16 pairs of cupules including two growth control wells and five antifungal drugs at different concentration. A suspension with a turbidity of 2 McFarland was prepared and 20 μl of this suspension was transferred to an ampule of ATB FUNGUS 3 Medium following the manufacturer’s instructions and 135 μl of the inoculated medium was transferred into each cupule. After incubation at 35°C for 24 h, the strips were read visually. According to the manufacturer’s instructions, the minimum inhibitory concentrations (MICs) were determined by the growth score for each of the cupules compared with the control cupules.

**Quality control strains**

*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as the control strains. Quality control was ensured by testing the CLSI-recommended quality control strains for ATB FUNGUS 3.

**RAPD analysis**

DNA extraction was carried out by means of the Biospin-Fungus Genomic DNA Extraction Kit (BioFlux, Shanghai, China). The concentration of nucleic acids was measured using a NanoDrop 2000 ultra-micro spectrophotometer. The primer was 5′-ACGGCCGACC-3′. Amplification reactions were carried out in an ABI 2700 thermal cycler (Applied Biosystems) with an initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 2 min, annealing at 36°C for 1 min, and extension at 72°C for 1 min, for 40 cycles, with a final extension at 72°C for 5 min. Each sample was run in duplicate. The resultant fragments of amplified DNA were analyzed by electrophoresis through 1.2% agarose gels run in 0.5× Tris-borate-EDTA buffer.
Gels were stained and photographed. Bands were scored based on presence/absence and not on intensity.

Definition of genomic groups
DNA patterns were compared using the NTsys 2.10e software and the similarity value ($S_{AB}$) was calculated for each pair of patterns on the basis of matching fragment positions. The cut-off value of $S_{AB}$ was 0.75 for genetic similarity. The $S_{AB}$ were then clustered by the unweighted pair group method using the arithmetic averages technique and dendrograms were generated to visualize the relationships between isolates.

Data analysis
The $\chi^2$ test was used for proportions in the statistical analysis, performed by means of GraphPad Prism version 5.0 for Windows (GraphPad Software, USA). $P < 0.05$ was considered a statistically significant difference.

Results
Strains
Among the 135 Candida strains, Candida albicans was the dominant pathogen found in vulvovaginal candidiasis patients (115, 85%) next followed by Candida glabrata (14, 10%), Candida tropicalis (3, 2%), Candida parapsilosis (2, 1.5%), and Candida krusei (1, 0.7%). Among the 115 Candida albicans isolates, 59 were isolated from patients in Obstetrics and Gynecology Hospital of Fudan University, 30 from International Peace Maternity and Child Health Hospital, and 26 from Shanghai First Maternity and Infant Hospital.

Antifungal susceptibility testing
In the present evaluation, ATB visual readings showed good concordance with CLSI microdilution susceptibility testing as described in the literature. According to the guidelines outlined in CLSI document M27S4, of the 115 Candida albicans strains, 95 (83%) were susceptible to fluconazole, and 93 (81%) were susceptible to voriconazole. (Table 1).

Table 1. Susceptibility rates of Candida albicans to the fungal drugs tested.

<table>
<thead>
<tr>
<th>Fungal agents</th>
<th>S (Sensitive)</th>
<th>SDD (susceptible Dose-Dependent)</th>
<th>R (Resistant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>83% (95/115)</td>
<td>10% (12/115)</td>
<td>7% (8/115)</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>81% (93/115)</td>
<td>5% (6/115)</td>
<td>14% (16/115)</td>
</tr>
</tbody>
</table>

Discussion
In our study, we found the Candida albicans strain to be the dominant pathogen causing vulvovaginal candidiasis (115, 85%) followed by Candida glabrata (14, 10%), Candida tropicalis (3, 2.2%), Candida parapsilosis (2, 1.5%), and Candida krusei (1, 0.7%). Among the 30 Candida albicans strains isolated from patients in International Peace Maternity and Child Health Hospital, 23 (76.7%) were classified as genotype A (including genotype A1–A4), 7 (23.3%) as genotype B (including genotypes B1 and B2). Of the 26 Candida albicans isolated from patients in Shanghai First Maternity and Infant Hospital, 21 (80.8%) were classified as genotype A (including genotype A1–A3, A5) and 5 as genotype B (19.2%) including genotypes B1 and B2. The distribution of RAPD types analyzed using a $\chi^2$ test showed no significant differences between the three maternity hospitals ($P > .05$).

Recently, researchers also reported that Candida albicans is the most common cause of vaginitis. In southern China, the most common pathogens causing vulvovaginal candidiasis reportedly were Candida albicans (91.4%), Candida glabrata (4.3%), Candida tropicalis (3.2%), and Candida parapsilosis (1.1%). In Argentina, the most
Figure 1. The clustering analysis of the electrophoretic bands of Candida albicans isolates. The cut-off value of $S_{AB}$ was 0.75 for genetic similarity.
common isolated yeast species causing vulvovaginitis was *Candida albicans* (85.2%) followed by *Candida glabrata* (5%), *Saccharomyces cerevisiae* (3.3%), and *Candida dubliniensis* (2.5%)\(^\text{19}\). In Kuwait, vaginal swab cultures of 231 women suffering from VVC yielded the following results: *Candida albicans* (73.9%), *Candida glabrata* (19.8%), *Candida kefiri* (1.94%), *Candida tropicalis* (0.96%), *Candida parapsilosis* (0.96%), *Candida krusei* (0.96%), *Candida guilliermondii* (0.96%), and *Saccharomyces cerevisiae* (0.52%).\(^\text{20}\)

*In vitro* antifungal susceptibility testing of *Candida albicans* is important to establish the susceptibility patterns of isolates recovered from different regions and countries to detect the prevalence of resistant isolates.\(^\text{21}\) Our analysis of *Candida albicans* antifungal susceptibility would be helpful in guiding effective clinical therapy regimes. Azole antifungals are the first line medication for candidiasis; compared with amphotericin B, these have low toxicity and elicit fewer adverse side effects. The susceptibility of *C. albicans* from different types of specimens and clinical services varied. A Taiwan study reported that the rates of fluconazole and voriconazole susceptibility were about 99% in blood *Candida albicans* isolates.\(^\text{22}\) The rate of fluconazole susceptibility was 91.1%, 78.6%, 87.3%, 85.3%, 90.9% for isolates from the respiratory, vaginal, urinary, digestive tract, and blood, respectively. Notably, the resistance rate for isolates from the vagina, burn patients, patients with infectious diseases and outpatients were very high. The high resistance rate in vaginal *C. albicans* isolates may be associated with repeated exposure to antifungal agents.\(^\text{23}\) In our study, the susceptibility rates of *Candida albicans* to fluconazole and voriconazole were about 80%. A 20% nonsusceptible rate to fluconazole and voriconazole may be because these 135 patients have a history of VVC, and the 7% resistant to fluconazole were collected from patients who had received prior fluconazole. The increased use of over-the-counter antifungals and prolonged therapy are risk factors for the emergence of azole resistance among *C. albicans* isolated from VVC patients. Recent exposure to azoles has been noted by others as an important risk factor for bloodstream infection with fluconazole-resistant *Candida* spp.\(^\text{24}\)

An investigation of the genetic relatedness between clinical *Candida* strains may be of great importance in the clinical diagnosis, epidemiology, treatment, and prevention of candidiasis. Molecular methods of studying strain relatedness have proven to be quite useful, allowing investigators to examine, retrospectively, putative outbreaks of candidiasis and assess epidemiological aspects of these outbreaks. RAPD analyses are used as tools useful in tracing the routes of transmission of different microorganisms, including *Candida* species.\(^\text{25}\)

In the present study, no significant differences were detected between the RAPD types in the three maternity hospitals. The majority of molecular typing was genotype A with genotype C only being isolated from patients in Obstetrics and Gynecology Hospital of Fudan University. Thus, unequivocally the genotype of *Candida albicans* in vaginal isolates from these institutions in Shanghai is mainly type A, although the RAPD technique raises the problem of reproducibility and data comparison between laboratories. This technique is still used to investigate *Candida albicans* epidemiology at the local level.\(^\text{26}\)

In conclusion, our study has shown that *Candida albicans* is the dominant pathogen of vulvovaginal candidiasis, and the increased use of azole antifungals may be an important risk factor for the emergence of azole resistance among *C. albicans* isolated from VVC patients. Molecular typing revealed that genotype A is predominant in this study. Our results provide experimental evidence for clinical application of azole agents and molecular epidemiology monitoring.

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**Declaration of interest**

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