RESEARCH ARTICLE

The changes of antifungal susceptibilities caused by the phenotypic switching of *Candida* species in 229 patients with vulvovaginal candidiasis

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Objective: This study was to investigate the changes of antifungal susceptibilities caused by the phenotypic switching in patients with vulvovaginal candidiasis (VVC).

Methods: 229 women were enrolled in this study. The vaginal smears of these patients were collected and gram stained for fungal microscopic observation. The vaginal discharge of them in cotton swabs was cultured in sabouraud's agar with chloramphenicol medium. After fungal culture, fungal identification was analyzed using CHROM agar *Candida* chromogenic and identification medium. Then, the in vitro antifungal susceptibility testing was carried out using the standardized CLSI M27-A2 broth microdilution method.

Results: 64.63% of *Candida* species in patients with VVC were *Candida albicans* and the remainders were *non-albicans Candida* species. The phenotypic switching was observed in 91.22% of *C. albicans* infection. In antifungal susceptibility testing, the susceptible rates of *C. albicans* to voriconazole, fluconazole and itraconazole were significantly higher than that of *non-albicans Candida* species ($P = 0.00, 0.00, 0.00$). No matters in patients infected with *C. albicans* or with *non-albicans Candida* species, the susceptible rate to fluconazole of the clinical isolates with phenotypic switching was significantly higher than that without phenotypic switching ($P = 0.01, 0.01$).

Conclusions: In the study, *C. albicans* was the commonest pathogenic species in patients with VVC, in which the phenotypic switching was easy to occur. The susceptible rates of *C. albicans* to all antifungal drugs were higher than that of *non-albicans Candida* species. The susceptible rate to fluconazole was all influenced by the phenotypic switching in *C. albicans* and *non-albicans Candida* species.

KEYWORDS

antifungal susceptibility testing, *Candida albicans*, *non-albicans Candida*, phenotypic switching, susceptible rate

1 | INTRODUCTION

Vulvovaginal candidiasis (VVC) is a common vaginal infection disease which is associated with significant morbidity and

unacceptable high recurrence rate.¹ *Candida* species is a well-known opportunistic pathogen of VVC, which includes *Candida albicans* and *non-albicans Candida* species. *C. albicans* is the commonest opportunistic pathogen in vagina, followed by *Candida glabrata*,

Abbreviation: AFST, Antifungal susceptibility testing; CAP, College of American Pathologists; CLSI, Clinical and Laboratory Standards Institute; ECVs, epidemiological cutoff values; IRB, Institutional Review Board; MIC, minimum inhibitory concentration; NCCLS, National Committee for Clinical Laboratory Standards; NSEQA, National System for External Quality Assessment; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; RVVC, recurrent vulvovaginal candidiasis; VVC vulvovaginal candidiasis
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Candida parapsilosis, *Candida tropicalis*, and so on.² However, the etiology of high VVC recurrence is *non-albicans Candida* species and the prevalence of other *Candida* species including *C. glabrata* is increasing in recent years.³ Moreover, up to 6% women will suffer from recurrent VVC (RVVC),⁴ which consists in acute and symptomatic individuals.⁵

Candida species is generally in yeast phase under the normal circumstances. In some cases, phenotypic switching will occur in *C. albicans*, which is from yeast phase to hypha phase.⁶⁻⁸ The phenotypic switching may lead to antigenic changes on the cell membrane, local immune response in mucosa, and the occurrence of vaginal inflammation and susceptibility to different antifungal drugs.⁹ This study was designed to investigate the changes of antifungal susceptibility testing caused by the phenotypic switching in patients with VVC, which was benefit for rational use of antifungal drugs.

2 | MATERIALS AND METHODS

2.1 | Samples collection

In this study, 229 patients were enrolled from the obstetrics and gynecology department of West China Second University Hospital, Sichuan University from January 2016 to November 2017. The basic information of participants is recorded including their age, pregnancy status, marital status, clinical symptoms, color, and amount of vaginal secretion, itching or dysuria of vulvar, past illness and treatment history before undergoing gynecologic examination. The inclusion criteria were as follows: (a) Women who suffered from vulva and vaginal itching. (b) The result of fungal culture and morphological examination was positive. (c) Presence or absence of abundant/cheesy vaginal discharge. Women of menstruation, mental disorder (could not answer the questions), those who taking medicines including antifungal drugs and/or topical vaginal creams within 7 days before examination and those who was diagnosed with RVVC (Who were diagnosed with VVC and their clinical symptoms and signs disappeared after treatment, then relapsed four or more times a year after negative mycological examination) were specifically excluded from the study. Vaginal discharge of these patients was collected by sterile cotton swabs (Medical Apparatus and Instruments Factory of Yangzhou Chuangxin, Jiangsu, China). A part of vaginal discharge on sterile cotton swabs was made into smears for fungal morphologic observation, the rest were used for fungal culture, identification, and antifungal susceptibility testing. In our study, the written informed consent was obtained from all participants and the privacy rights of participants were also reserved. And all procedures and protocols are in accordance with the Helsinki Declaration as revised in 2013. The study protocol was approved by the Institutional Review Board (IRB) of the West China Second University Hospital, Sichuan University before study initiation.

2.2 | Gram staining and microscopic observation

Vaginal smears of these 229 patients were subjected to gram staining. The exact staining procedure may refer to related literature by Dai et al¹⁰ After gram staining, the presence or absence of gram positive yeasts with/or without budding and/or pseudohyphae in vaginal smears were observed under microscope. It is reported that the formation of hyphae or pseudohyphae is the symbol of fungal phenotypic switching.¹¹ *Candida* species were identified by two laboratory microbiologists under the oil immersion field microscope based on the National System for External Quality Assessment (NSEQA) and the College of American Pathologists (CAP).¹²

2.3 | Fungal culture

The vaginal discharge in cotton wool-tipped swabs was cultured in sabouraud's agar with chloramphenicol medium (Jinzhong science and technology development limited company, Tianjin). After incubation at 37°C for 2-3 days, the gray-white cheese colonies formed. Ovoid yeasts and pseudohyphae were also observed after gram staining. The colony would be regarded as *C. albicans* or *non-albicans Candida* species.

2.4 | Fungal identification and in vitro antifungal susceptibility testing

After fungal culture, fungal identification was analyzed using CHROM agar *Candida* chromogenic and identification medium (CHROM agar Company, France). Only one isolate had been identified from per patient. So, there were 229 clinical isolates were initially identified in this study. When the results of fungal culture were positive and the isolates were identified as *C. albicans* or one of *non-albicans Candida* species, the susceptibilities of *Candida* species to azole antifungal drugs (fluconazole, voriconazole, itraconazole) and 5-Flucytosine were determined using the standardized CLSI M27-A2 broth microdilution method with the following testing guidelines: (a) RPMI 1640 with 2% dextrose medium to enhance the growth of yeast cells; (b) an inoculum size of 0.5×10^5 to 2.5×10^5 CFU/mL; (c) flat-bottom microdilution trays; and (d) 24-hour spectrophotometric MICs.¹³ Important new developments including validation of 24-hour reading times for all antifungal agents and the species-specific epidemiological cutoff values (ECVs) were established Conversely, ECVs can be considered to represent the most sensitive measure of the emergence of strains with decreased susceptibility to a given agent.¹⁴ The interpretive breakpoints for susceptibility to azoles were those employed by Sanguinetti et al¹⁵ and were as follows: (a) fluconazole, ≤ 8 mg/L, sensitive; ≥ 64 mg/L, resistant; (b) voriconazole, ≤ 1 mg/L, sensitive; ≥ 4 mg/L, resistant; (c) itraconazole, ≤ 0.125 mg/L, sensitive; ≥ 1 mg/L, resistant; (d) 5- Flucytosine, ≤ 4 mg/L, sensitive; ≥ 32 mg/L, resistant. The susceptibilities of isolates to amphotericin B were assayed as described previously.¹⁶ The minimum inhibitory concentration (MIC) for amphotericin B was defined as the lowest drug concentration at which growth was completely inhibited.¹⁷

2.5 | Statistical analysis

The data analysis was performed by SPSS Statistics ver. 17.0 (SPSS Inc., Chicago, IL, USA). The comparison of different prevalence among *Candida* species and the different positive rates of phenotypic switching were analyzed by Chi-squared test. A 2-tailed $P < 0.05$ was considered statistically significant and a 2-tailed $P < 0.01$ was considered extremely statistically significant.

3 | RESULTS

3.1 | Results of microscopic observation and fungal identification

In CHROM agar *Candida* chromogenic and identification mediums, the colonies were shown in Figures 1 and 2. The colony color of *C. albicans* was green (diameter: 2 mm), while the colony color of *C. glabrata* was purple (diameter: 2 mm). The colony color of *C. krusei* and *C. tropicalis* was soft pink (diameter: 4–5 mm) and blue gray or iron blue (diameter: 1.5 mm) respectively. The colony color of *C. parapsilosis* was white.

According to the colony morphology, 64.63% of *Candida* species in patients with VVC were identified as *C. Albicans* ($n = 148$) and the remainders ($n = 81$, 35.37%) were identified as *non-albicans Candida* species (showed in Table 1). In the clinical isolates identified as *non-albicans Candida* species, 91.36%, 2.47%, 2.47%, 2.47%, and 1.23% of them were identified as *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, and *Saccharomyces cerevisiae* respectively. Although we have not used the molecular method to identify the *Candida* species, instead of using CHROM agar *Candida* chromogenic and identification medium, the results of our study were consistent with the results of Hasanvand et al.¹⁸ and Seyed Amir Y et al.¹⁹

Based on related literature by Ilkit et al.,¹¹ after gram staining, the finding of pseudohyphae was regarded as phenotypic

FIGURE 1 The colors of *Candida* species in CHROMagar *Candida* chromogenic and identification medium. The color of green and purple represents *C. albicans* and *C. glabrata*, respectively; the color of soft pink and blue-gray represents *C. krusei* and *C. tropicalis*, respectively

FIGURE 2 The colors of two *Candida* species in CHROMagar *Candida* chromogenic and identification medium. The color of iron blue and white represents the *C. tropicalis* and *C. parapsilosis*, respectively

switching (Figure 3A). And the finding of yeasts without budding (without pseudohyphae) was regarded as non-phenotypic switching (Figure 3B). The yeasts with budding and pseudohyphae were observed in 91.22% of clinical isolates identified as *C. albicans*, which indicated that 91.22% of these clinical isolates were with phenotypic switching. The remainder (8.78%) was without phenotypic switching. Meanwhile, the pseudohypha was not observed in 80.25% of clinical isolates identified as *non-albicans Candida* species, which indicated that 80.25% of these clinical isolates was without phenotypic switching, the remainders (19.75%) was with phenotypic switching (showed in Table 1).

3.2 | Results of in vitro antifungal susceptibility testing

In antifungal susceptibility testing in vitro, the susceptible rates to amphotericin B, 5-fluorocytosine, voriconazole, fluconazole, and itraconazole of *C. Albicans* isolates with and without phenotypic switching were listed in Table 1. The susceptible rates to the above five drugs of *non-albicans Candida* isolates with & without phenotypic switching and the difference of the susceptible rate between *C. Albicans* isolates and *non-albicans Candida* isolates were also listed. The susceptible rates of *C. albicans* isolates to voriconazole, fluconazole, and itraconazole were higher than that of *non-albicans Candida* isolates ($P = 0.00, 0.00, 0.00$). No matters for *C. Albicans* isolates and *non-albicans Candida* isolates, the susceptible rate to fluconazole with phenotypic switching was significantly higher than that without phenotypic switching ($P = 0.01, 0.01$).

TABLE 1 Antifungal susceptibility testing of different *Candida* species and the difference of drug-susceptible rates between *Candida* isolates with and without phenotypic switching

	The percentage of drug-susceptible cases and the susceptible difference between isolates with and without phenotypic switching					
	Positive rates (Cases/total cases)	Amphotericin B (% χ^2 , <i>P</i>)	5-Fluorocytosine (% χ^2 , <i>P</i>)	Voriconazole (% χ^2 , <i>P</i>)	Fluconazole (% χ^2 , <i>P</i>)	Itraconazole (% χ^2 , <i>P</i>)
<i>Candida albicans</i>	64.63 (148/229)	148 (100%)	148 (100%)	132 (89.20%)	121 (81.76%)	111 (75.00%)
With phenotypic switching	91.22% (135/148)	135 (100%)	135 (100%)	123 (91.11%)	114 (84.44)	98 (72.59%)
Without phenotypic switching	8.78% (13/148)	13 (100%)	13 (100%)	13 (100%)	7 (58.35%)	9 (69.23%)
<i>Non-albicans candida</i> (NAC)	35.37% (81/229)	80 (98.77%)	79 (97.53%)	58 (71.60%)	47 (58.02%)	20 (24.69%)
With phenotypic switching	19.75% (16/81)	16 (100%)	15 (93.75%)	12 (75.00%)	9 (56.25%)	5 (31.25)
Without phenotypic switching	80.25% (65/81)	64 (98.46%)	64 (98.46%)	46 (70.77%)	16 (24.62%)	15 (23.08%)

^aThe difference of drug-susceptible rates between *Candida* isolates with phenotypic switching and *Candida* isolates without phenotypic switching

^bThe difference of drug-susceptible rates between *C. albicans* isolates and *non-albicans candida* isolates.

4 | DISCUSSION

In this study, through fungal morphology observation and fungal identification, *C. albicans* was found to be the main pathogen of VVC. This is consistent with the findings in previous studies.²⁰⁻²² *C. glabrata* was the commonest pathogenic species in *non-albicans Candida* species, followed by *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *Saccharomyces cerevisiae*, which was supported by other reports.^{2,23} Furthermore, VVC caused by *Saccharomyces cerevisiae* has been firstly reported in China or other countries.²⁰

The yeasts with budding and pseudohyphae were observed in 91.22% of *C. albicans* isolates, in which the fungal phenotypic switching had occurred. Meanwhile, the pseudohyphae was not observed in 80.25% of *non-albicans Candida* isolates, in which the phenotypic switching did not happen. This indicated that the infection of *C. albicans* was more prone to fungal phenotypic switching than that of *non-albicans Candida* species.²⁴ Meanwhile, it was the first time to find that yeasts with budding and pseudohyphae were observed in 19.75% of clinical isolates identified as *non-albicans Candida*, which indicated that 19.75% of these clinical isolates was with phenotypic switching.

On account of the immunological mechanisms, fungal phenotypic switching can cause the changes of *Candida* antigens. The phenotypic switching of *Candida* species from yeast phase to hypha phase may cause the topical mucosal immunological responses and strengthen the invasiveness capacity of toxicity to host. The hyphal formation of *Candida* species is the main symbol of phenotypic switching.^{11,21} The hyphae can grow along the fissure of skin and mucosa by mechanical force and finally pass through the skin or skin epithelial cells, which are two important steps of fungal infection.²⁵

In antifungal susceptibility testing, the susceptible rates of *C. albicans* to all antifungal drugs were higher than *non-albicans Candida* species (Tab. 1). The susceptible rates of *C. albicans* to voriconazole, fluconazole and itraconazole were significantly higher than that of *non-albicans Candida* species, which indicated that the commonly used antifungal drugs aimed to VVC caused by *C. albicans* were more effective than that caused by *non-albicans Candida* species. Patients infected with *non-albicans Candida* species have various degrees of resistance to different antifungal drugs.¹¹ Azole antifungals are efficient in clearing the acute infection but unable to prevent the recurrence of VVC. So, it should be careful when using these two kinds of drugs.²⁶

Fungal phenotypic switching was associated with the susceptibility of some antifungal drugs. In immune activation state, fungal hyphae are more easily inhibited or destructed by azole antifungals such as fluconazole.²⁷ Therefore, azole antifungals have better therapeutic effect against VVC caused by *C. albicans*. *Non-albicans Candida* species is often in the form of yeasts without budding and phenotypic switching occurred rarely on their fungal cell membrane. The antigenicity does not change and is immunology suppressed. Yeasts has far lower toxicity and invasion to the body than fungal hyphae do. The conventional antifungal drugs such as fluconazole did not inhibit or damage the yeasts. So, the susceptible rates of *non-albicans Candida* species to these antifungal drugs are decreased. Meanwhile, the drug resistance

FIGURE 3 A, the presence of pseudohyphae under microscopic observation (with phenotypic switching) (Gram staining, 1000 $\times$); B, the presence of yeasts without budding under microscopic observation (without phenotypic switching) (Gram staining, 1000 $\times$)

spectrum increased, which is further increased with the overuse of antifungal drugs.²⁸

In this study, the susceptible rate to fluconazole of *C. Albicans* isolates with phenotypic switching was significantly higher than that without phenotypic switching. It was mainly because that fungal phenotypic switching can cause the change of *Candida* antigens, leading to the rise of susceptibility to azole antifungal such as fluconazole. Meanwhile, the susceptible rate to fluconazole of *non-albicans Candida* species with phenotypic switching was still significantly higher than that without phenotypic switching. However, whether phenotypic switching occurred or not, there was no significant difference of the susceptible rates to other drugs such as amphotericin B, 5-fluorocytosine, itraconazole, and voriconazole in *Candida* isolates.

In summary, *C. albicans* was the commonest pathogenic species of VVC, which was easy to convert from yeast phase to hypha phase. Meanwhile, the phenotypic switching was rarely occurred in *non-albicans Candida* species. The susceptible rate of *C. albicans* to antifungal drugs was higher than that of *non-albicans Candida* species. No matters for patients infected with *C. albicans* or with *non-albicans Candida* species, the susceptible rates to fluconazole with phenotypic switching was significantly higher than that without phenotypic switching. So, the rational use of fluconazole would be based on the fungal phenotypic switching in patients with VVC.

Our study has some limitations. First, the sorts of antifungal drugs for susceptibility testing were quite less, which could not completely represent all antifungal drugs. Therefore, newer antifungal drugs will be used in our future studies. Second, in patients who infected with *non-albicans Candida* species, regardless of cases with or without phenotypic switching, the study number was relatively small, which may lead to statistical error to an extent. More cases will be enrolled in our future study. Third, although the antifungal susceptibilities of different *non-albicans Candida* species such as *C. glabrata* and *C. parapsilosis* are different, however, because of the small number and proportion of *C. krusei*, *C. tropicalis*, *C. parapsilosis*, and *Saccharomyces cerevisiae* cases, the susceptibilities of all *non-albicans Candida* isolates had to be counted together in our study. Furthermore, molecular method such as PCR-RFLP analysis would be used to identify the *Candida* species instead of using CHROM agar *Candida* chromogenic and identification mediums.^{17,18}

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