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Epidemiology, species distribution, antifungal susceptibility, and *ERG11* mutations of *Candida* species isolated from pregnant Chinese Han women

L. Yang, M.Q. Su, Y.Y. Ma, Y.J. Xin, R.B. Han, R. Zhang, J. Wen and X.K. Hao

Center for Clinical Laboratory Medicine of PLA, Xijing Hospital, Fourth Military Medical University, Xi'an, China

Corresponding author: X.K. Hao
E-mail: haoxiaoke2015@163.com

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ABSTRACT. The widespread use of antifungal agents has led to increasing azole resistance in *Candida* species. A major azole-resistance mechanism involves point mutations in the *ERG11* gene, which encodes cytochrome P450 lanosterol 14a-demethylase. In this study, vaginal swabs were obtained from 657 pregnant Chinese Han women and cultured appropriately. The open reading frame of the obtained fungal species were amplified by PCR and sequenced; additionally, the *ERG11* gene of the isolated *Candida* species was amplified and sequenced, and the antifungal susceptibility of the isolated species was determined. The vaginal swabs of 124 women produced fungal cultures; five species of *Candida* were isolated from the patients, among which *Candida albicans* was predominant. Twelve *C. albicans* isolates (13.8%) were resistant to fluconazole and 2 (2.2%) were resistant to itraconazole. Seventeen mutations, including 9 silent and 8 missense mutations, were identified in the *ERG11* gene of 31 *C. albicans* isolates. Our findings

suggest that infection caused by *C. albicans* and non-*C. albicans*is common in Chinese Han women of reproductive age. Moreover, the relationship between *Candida* infection and certain epidemiological factors emphasizes the need to educate women about the precise diagnosis and punctual treatment of vaginitis.

Key words: Antifungal susceptibility; *Candida* species; *ERG11*

INTRODUCTION

Candida species remains the most common cause of human infections (Richardson and Lass-Flörl, 2008). Diabetes mellitus, broad-spectrum antibiotic use, pregnancy, and immunodeficiency are some of the important risk factors for Candida genital infection (Mascarenhas et al., 2012). Reports have suggested that approximately 75% of women over 25 years of age complain of at least one episode of physician-approved vulvovaginal candidiasis (VVC) during their lifetime, with at least 5% of these cases recurring at least 4 times in a one-year period. However, asymptomatic microorganism colonization can occur in 25 to 50% of the cases (Mahmoudi Rad et al., 2012; Mascarenhas, et al., 2012). *Candida albicans* is the most common and clinically relevant pathogen that is responsible for 85-90% of the VVC cases (Mahmoudi Rad et al., 2012). However, other species of *Candida*, including *Candida dubliniensis*, *Candida glabrata*, *Candida krusei*, and *Candida parapsilosis*, have been the focus of increasing research over the past few years, due to the development of resistance to first line antifungal treatment strategies (Tortorano, et al., 2004).

Preterm birth (PTB) is usually defined as delivery prior to 37 gestation weeks (the normal gestation period is 40 to 42 weeks). Premature or preterm birth is the leading cause of neonatal mortality and morbidity in newborns without congenital anomalies or chromosomal abnormalities (Mathews and MacDorman, 2006; Hamilton et al., 2013). It is also associated with a broad spectrum of lifelong neonatal mortality and long term morbidities, such as respiratory problems, learning difficulties, cerebral palsy, and behavior problems (Costeloe, et al., 2012). Despite the major advances in basic clinical and translational research and the development of medical intervention strategies to reduce PTB, the incidence rate of PTB (11.7% in the United States in 2011) has remained relatively stable over the past few decades (Raju, 2006). PTB is the most pressing problem faced by obstetricians today; however, incomplete understanding of its risk factors has impeded the development of new and effective prevention and treatment strategies for PTB. An estimated 50% of all spontaneous preterm births are associated with ascending genital tract infection, particularly those occurring before 30 weeks of gestation (Lockwood and Kuczynski, 1999; Challis et al., 2001). Moreover, bacterial infection of the chorioamnion in preterm infants has been associated with preterm birth, histologic chorioamnionitis, and neonatal death (Hillier et al., 1991).

Several observational studies have explored the association between candidiasis and preterm birth. A previous randomized control trial of early antenatal screening (15-20 weeks) and treatment for asymptomatic candidiasis, bacterial vaginosis, and/or trichomoniasis in early pregnancy reported a 46% decrease in the rate of spontaneous PTB (Kiss et al., 2004). *Post-hoc* subgroup analysis of this trial revealed that the benefit was primarily in those women treated for asymptomatic candidiasis. A similar decrease in occurrence of PTB (49%) was observed in a retrospective study of Hispanic women in New York treated with intravaginal

azoles for *Candida* vaginitis (Morrison and Cushman, 2007). Hence, treatment of vaginal candidiasis, especially asymptomatic vaginal candidiasis, during pregnancy might prevent PTB.

The purpose of this study was to determine the prevalence and antimicrobial susceptibility of *Candida* species isolated from pregnant women, and to explore the association between *ERG11* mutations in *Candida* species and antifungal drug resistance.

MATERIAL AND METHODS

Study population

Six-hundred and fifty-seven pregnant women aged 21-43 years at gestation weeks 30-35, who underwent routine prenatal care at the Gynecological Clinic at the Xijing Hospital, Fourth Military Medical University between September 2012 and April 2013 were enrolled in this study. Women taking antifungal agents at least two weeks prior to the study, or those with preexisting medical disorders complicating pregnancy, were excluded from the study. This study was approved by the Research and Ethical Committees of the Xijing Hospital, Fourth Military Medical University; informed consent was obtained from all included patients. Initial baseline data was collected when vaginal swab samples were taken (self-collection) from all patients for the isolation of *Candida* species.

Isolation of *Candida* species

The vaginal swabs were cultured immediately on selective Sabouraud dextrose agar at 37°C for 24 h. Isolates were characterized using the API20C AUX kit (bioMerieux, Marcy-l'Étoile, France), CHROMagar *Candida* (bioMerieux), and via germ tube formation. Pure isolates were stored in brain heart infusion broth with 10% glycerol at -80°C.

Antifungal susceptibility of *Candida* species

Susceptibility of *Candida* isolates to the antifungal agents amphotericin B, fluconazole, and itraconazole were determined using the ATB™ Fungus 3 test kit (bioMerieux) according to manufacturer protocols. Fungal suspensions were adjusted to a 2McFarland turbidity standard. The readings were obtained after 24-48 h of incubation at 37°C. *C. parapsilosis* NCTC 3104 was used as the quality control. Antifungal susceptibility results were analyzed using the interpretive criteria recommended by the Clinical and Laboratory Standard Institute (CLSI) for fluconazole and itraconazole, as described in a previous study (Moris, et al., 2012). Samples with a minimum inhibitory concentration (MIC) of amphotericin B that were £1 mg/L were considered to be susceptible (CLSI guidelines of 2002).

DNA amplification

Genomic DNA was extracted from *Candida* species with a Biospin Fungus Genomic DNA Extraction kit (Bioer Technology, Hangzhou, China) according to the manufacturer instructions. PCR was performed using primers spanning the complete open reading frame of *ERG11*: 5'-GTT GAA ACTGTC ATT GAT GG-3' (forward) and 5'-TCA GAA CACTGA ATC GAA AG-3' (reverse) (Pam, et al., 2012). PCR was performed in an S-1000 thermal

cycler (Bio-Rad, Hercules, CA, USA) under the following reaction conditions: denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 43°C for 60 s, and extension at 72°C for 60 s; and a final extension at 72°C for 10 min. The reference strain *C. parapsilosis* ATCC22019 was also included in each run. Positive PCR products were sequenced by Shanghai Shengong Biotechnology Co. (Shanghai, China).

RESULTS

The mean age (±standard deviation) of the patients was 29.3 (±9.5) years: 26.9% of the pregnant women were overweight, 15.1% used broad spectrum antibiotics, 4.9% were diabetic, and 17.0% used vaginal douching (Table 1).

Table 1. Demographic and baseline clinical characteristics of pregnant Chinese Han women included in this study.

Variables	Total participants N (%)
N	657
Age (years)	
21-25	132 (20.1)
26-30	221 (33.6)
31-35	189 (28.8)
36-40	83 (12.6)
41-45	32 (4.9)
BMI > 25	177 (26.9)
Diabetic	32 (4.9)
Antibiotic utilization	99 (15.1)
Vaginal douching	112 (17.0)

BMI = body mass index.

Five species of *Candida* were isolated from 124 women; *C. albicans* was the species with the highest isolation frequency (70.1%). The other species isolated from the patients were *C. tropicalis* (12.1%; most prevalent), *C. glabrata* (9.7%), *C. parapsilosis* (4.8%), and *C. krusei* (3.2%; Table 2). The women included in the study were aged between 21 and 43 years. The results of this study showed that women in the age group 26-30 were predominant lypositive for *Candida* infection, followed by those in the 31-35 age group; on the other hand, women aged above 41 years were the least susceptible to *Candida* infection (Table 2).

Table 2. Species distribution of *Candida* in pregnant women from different age groups.

	Candida albicans [N (%)]	*Candida tropicalis* [N (%)]	*Candida glabrata* [N (%)]	*Candida parapsilosis* [N (%)]	*Candida krusei* [N (%)]
21-25	17	2	1	0	0
26-30	29	5	5	3	2
31-35	25	5	3	2	2
36-40	11	2	2	1	0
41-45	5	1	1	0	0
Total	87 (70.1)	15 (12.1)	12 (9.7)	6 (4.8)	4 (3.2)

Total number of *Candida*-positive samples = 124.

The number of *Candida* isolates (obtained from pregnant Chinese Han women) determined to be susceptible, susceptible-dose dependent (S-DD), and resistant (R) to fluconazole, itraconazole, and amphotericin B are listed in Table 3. *C. albicans* was predominantly isolated. Twelve isolates (13.8%) were resistant to fluconazole, while 2 (2.2%) were resistant to itraconazole. Of the 15 *C. tropicalis* strains, 9 (60.0%) were sensitive to

fluconazole and 13 (86.7%) were sensitive to itraconazole. Eight (66.7%) among the 12 *C. glabrata* isolates were sensitive to fluconazole; all of these isolates (100%) were sensitive to itraconazole. *C. parapsilosis* isolates were highly sensitive to all antifungal agents, while *C. krusei* was least sensitive to fluconazole 2 (50.0%), and highly sensitive (100%) to the other antifungal agents. All isolates were susceptible to amphotericin B (Table 3).

Table 3. Antifungal susceptibility of *Candida* strains isolated from pregnant women.

		Candida albicans	*Candida tropicalis*	*Candida glabrata*	*Candida parapsilosis*	*Candida krusei*
Fluconazole	S	65	9	8	6	2
	SDD	10	6	4	0	2
	R	12	0	0	0	0
Itraconazole	S	72	13	12	6	4
	SDD	13	2	0	0	0
	R	2	0	0	0	0
Amphotericin B	S	87	15	12	6	4
	SDD	0	0	0	0	0
	R	0	0	0	0	0

S = susceptible; SDD = susceptible-dose dependent; R = resistant.

Seventeen mutations, including 9 silent and 8 missense mutations, were observed in the *ERG11* PCR product sequences of 31 *C. albicans* isolates. Eight missense mutations were identified in the resistant isolates (Table 4). A G487T mutation was observed in 8 resistant isolates: CA11, CA25, CA26, CA42, CA63, CA66, CA81, and CA82. This mutation was absent in the *ERG11* sequences of the other isolates. The resistant isolate CA6 also displayed three missense mutations T495A, A530C, and T541C. The resistant isolate CA19 also contained three missense mutations (T495A, A530C, and T1493A). While 2 missense mutations were detected in the resistant isolate CA37 (T495A and A530C), the resistant isolate CA42 showed three missense mutations: T495A, A530C, and C1567A. Another resistant isolate CA73 displayed two missense mutations A530C and C1567A.

Table 4. Missense mutations in *ERG11* of resistant isolates of *Candida albicans*.

Isolate name/number	Amino acid substitution	Nucleotide substitution
Eight isolates*	A114S	G487T
CA6	D116E K128T Y132H	T495A A530C T541C
CA19	D116E K128T F449Y	T495A A530C T1493A
CA37	D116E K128T	T495A A530C
CA42	D116E K128T Q474K	T495A A530C C1567A
CA73	K128T Q474K	A530C C1567A

*Eight isolates were CA11, CA25, CA26, CA42, CA63, CA66, CA81, and CA82.

DISCUSSION

Although the fungus *C. albicans* is a commensal colonizer of humans, it is also an important opportunistic pathogen that can infect a broad range of sites in the body. Pregnancy is a major risk factor of vaginal candidiasis. The aim of our study was to detect the frequency of infection of pregnant women with *Candida* species, and to determine the frequency distribution of *C. albicans* and other *Candida* species. The results of this study showed that 18.9% of the participants were infected with different species of *Candida*. Tchelougou et al. (2013) attempted to determine the prevalence of major pathogens in pregnant women with

vaginal infections consulting at the Regional Hospital of Sokodé. They discovered that 30.77% of the participants were infected with *Candida* (Tchelougou et al., 2013). Babic and Hukic (2010) reported that pregnant women were predominantly infected with *C. albicans* (40.9%). The result of this study, however, suggested that very few pregnant Chinese Hanwomen were infected with *Candida*. However, these results must be validated with further research.

The swab samples from approximately one-fifth of the 657 participants (124) yielded *Candida* isolates. The *Candida* species *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei* were isolated from the swabs: *C. albicans* was predominant lyisolated (46.9%), followed by *C. tropicalis*. These results are in accordance with the results of previous studies, wherein *C. albicans* was the most common isolate, with a prevalence rate of 20.1-90%; however, in these studies, *C. glabrata* followed *C. albicans*, as the most prevalent (18-37%) (Okungbowa et al., 2003; Lopes Consolaro et al., 2004; Pirotta and Garland, 2006). Similarly, *C. albicans* and *C. glabrata* were predominantly isolated from samples collected in Iran (Mahmoudi Rad et al., 2011). The differences between the results of this study and those of previous studies could be attributed to geographic and ethnic factors. The prevalence of other *Candida* species was similar in pattern to those observed in earlier studies (Kunzelmann et al., 1996; Abu-Elteen, 2001).

Antifungal susceptibility of clinically important *Candida* species must be determined in order to design appropriate strategies for empiric and prophylactic therapies. Fluconazole is the first drug of choice for the treatment of vaginitis in most parts of the world in accordance with recommended guidelines (Maertens, 2004). However, fluconazole resistance has been reported in both *C. albicans* and non-*albicans* species (Clancy et al., 2005; Liu et al., 2009). Approximately 11.5% of the isolates identified as *C. albicans* were found to be susceptible (in a dose-dependent manner), and 12 isolates were found to be resistant, to fluconazole. The overall resistance rate (11.3%) observed in this study was similar to that observed in previous studies (9.5%) conducted in patients with HIV (Enwuru et al., 2008; Hamza et al., 2008) and genitourinary tract (Von Akortha and Chikwe, 2009) infections. Itraconazoleis an important drug of choice for the treatment of *Candida vaginitis*. However, it is not the first-line of treatment in China because of its high cost; it is usually prescribed after the failure of fluconazole in treating vaginitis. Therefore, a low resistance against this drug is to be expected. The widespread use of antifungal drugs to combat serious fungal infections has led to an increase in resistance to antifungal agents. Therefore, we highlight the need for routine susceptibility testing before the prescription of antifungal agents.

Mutations in the *ERG11* gene of *C. albicans* was also analyzed in the 12 clinical isolates with reduced susceptibility to fluconazole, using PCR amplification and gene sequencing. This analysis identified 6 previously reported amino acid substitutions (G487T, T495A, A530C, T541C, T1493A, and C1567A). No other mutations were detected in the 8 isolates except G487T (A114S), which differs from most other reports with regards to the accordance of many isolates. Moreover, the 8 isolates could be derived from the same resistant strain in the population. The A114S and Y257H substitutions have been reported to occur simultaneously in 14 isolates resistant to fluconazole (Xu et al., 2008); here, we observed the same phenomenon in 12 resistant isolates. This implies that G487T and/or T916C might be correlated with fluconazole resistance in *C. albicans*. However, the amino acid substitutions D116E and V437I shown by Xiang et al. (2013), as well as the Y205E substitution described in this study were found in both azole-susceptible and -resistant strains, strongly suggesting that these were not associated with the azole-resistant phenotype. Moreover, the occurrence

of K143R, G464S, G465S, S412T, V488I, and R469K mutations have also been reported in the *ERG11*gene, which were confirmed to affect the susceptibility mechanisms in fluconazole R/S-DD *C. albicans* isolates (Manastır et al., 2011).

In summary, infections caused by *C. albicans* and non-*C. albicans* species are common in women of reproductive age. The relationship between *Candida* infection and certain epidemiological factors emphasizes the need to educate women about the precise diagnosis and punctual treatment of vaginitis.

Conflicts of interest

The authors declare no conflict of interest.

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