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Genotyping Reveals no Link Between *Candida albicans* Genotype and Vaginitis Severity in Turkish Women

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Abstract Recent studies have clearly defined the vaginopathic *Candida albicans* strains that cause severe vulvovaginal candidiasis (VVC). Therefore, genotyping *C. albicans* isolates may predict the success of and assist in choosing the appropriate antifungal therapy. The purpose of this study was to compare the genotypes of *C. albicans* isolates causing VVC with those found in asymptomatic healthy pregnant and non-pregnant women in Adana, Turkey, as well as the antifungal susceptibility profiles of these isolates. A total of 216 independent *C. albicans* isolates were genotyped by allelic combination based on the microsatellite marker analysis of one such microsatellite, present in the promoter region of the elongation factor 3-encoding gene (*CEF3*) of *C. albicans*. The susceptibility testing profiles of all of the isolates against five antifungals and boric acid were obtained retrospectively from our laboratory records. We identified 20 genotypes on the basis of different allelic combinations at the *CEF3* locus with a discriminatory power of 0.85. Genotypes 136–144 and 126–135 were present in 50 % of the isolates. No differences existed in the genotypic profiles of fungal isolates between pregnant and non-pregnant women. Remarkably, we did not find a single vaginopathic genotype. All of the isolates were susceptible to amphotericin B and 5-fluorocytosine, and the fluconazole and ketoconazole resistance rates were 0.9 and 3.7 %, respectively. Therefore, we did not find any correlation between genotype, severity of VVC, and antifungal resistance ($P > 0.05$). Even so, additional molecular data may provide new insights into the management of VVC.

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Introduction

Vulvovaginal candidiasis (VVC) is a common infection, affecting up to 75 % of women of childbearing age at least once in their lifetime. *Candida albicans* is the dominant species that causes VVC, with a prevalence of 70–90 % [1–3]. VVC manifests in two forms: acute and recurrent. Acute VVC (AVVC) is characterized by sporadic or infrequent infection episodes (≤3 episodes per year), with mild-to-moderate symptoms caused by *Candida* spp., particularly *C. albicans*. AVVC is responsive to all forms of therapy (including short course) in immunocompetent women [1, 2]. The second form, recurrent VVC (RVVC), is defined as ≥4 culture-verified symptomatic episodes of VVC within 1 year, and it is caused by azole-susceptible *C. albicans* [1, 2] or, more often, azole-resistant non-*C. albicans Candida* species, for example, *C. glabrata* [4]. Based on the severity of symptoms, frequency of infection, and particular etiologic agent, VVC is usually classified as either uncomplicated or complicated. Uncomplicated VVC (mild and sporadic) occurs in normal, non-pregnant hosts. In contrast, complicated VVC is defined as follows: (1) cases of severe VVC, (2) cases of VVC associated with pregnancy or other concurrent conditions, for example, uncontrolled diabetes mellitus and immunosuppression, and (3) cases of RVVC in immunocompetent women [1–5].

Genotyping vaginal *C. albicans* isolates based on RAPD analysis [6, 7] or PCR fingerprinting [8, 9] has failed to show an obvious relationship between specific genotypes and VVC or RVVC groups [6–8] or their geographical origins [9]. However, via microsatellite typing [10–13] or MLST [13], the existence of vaginopathic *C. albicans* strains with enhanced virulence and tropism for the vagina has been revealed (in several studies based in China) [10–13], suggesting that *C. albicans* is sexually transmitted [11]. Microsatellite typing method has been used to identify, by genotype, vaginopathic isolates that are common in severe VVC cases but uncommon among other clinical isolates [10–13]. Other studies have also shown the importance of the geographical origin of the isolates, for example, isolates of Chinese women are different from the isolates of women from the rest of the world [12, 13]. The identification of specific genotypes that correlate with the severity of VVC has diagnostic and therapeutic implications [11]. Furthermore, only a few studies have focused on the genetic diversity of the *C. albicans* strains recovered from VVC patients and the correlation between antifungal susceptibility and *C. albicans* genotype [14–19].

The treatment for VVC usually involves the topical application of azole or polyene agents [20]. Vaginal *C. albicans* isolates are infrequently fluconazole (FLU)-resistant [14, 21, 22]. However, non-*C. albicans Candida* species are more resistant, possibly due to the prevalent use of over-the-counter azole agents, including FLU [22]. Hence, determining the choice of antifungal therapy based on in vitro susceptibility has had limited success, and this strategy may be correlated with poor clinical outcomes [21, 23, 24]. The variable host response to treatment and an unpredictable fungal load in the vulvovaginal mucosa (*in loco*) invariably weaken in vitro and in vivo correlations [18]. For example, a decrease in FLU susceptibility or a low FLU concentration in the vagina is likely related to FLU therapeutic failure [23, 24].

Molecular markers for the study of the epidemiology of *C. albicans* vaginitis are highly valuable to the clinical management of *C. albicans* infections, but no studies are available on the use of such markers in Turkey. Thus, for this study, *CEF3* microsatellite loci were used to identify polymorphisms in microsatellite length to conduct the genotyping. This locus was selected because of its high discriminatory power (DP; 0.87–0.97) [25, 26]. Because *C. albicans* is a diploid organism and the *CEF3* gene is a single-copy locus in the *C. albicans* genome, one or two distinct PCR fragments (representing alleles) may result from the amplification of the *CEF3* microsatellite in homozygous or heterozygous isolates, respectively [25]. In this investigation, we aimed to (1) determine through *CEF3* microsatellite marker analysis the genetic diversity of *C. albicans* isolates recovered from healthy asymptomatic women or those that cause complicated or uncomplicated vaginitis and (2) correlate the antifungal susceptibility and genetic diversity of these *C. albicans* isolates.

Materials and Methods

Clinical Presentations and *C. albicans* Isolates

A total of 216 independent vaginal *C. albicans* stock isolates that were recovered from pregnant ($n = 110$) and non-pregnant ($n = 106$) patients visiting the Obstetrics and Gynecology Department, Faculty of Medicine, Çukurova University, from 2010 to 2012 were used in this study. The definitions for the clinical presentations of VVC for each group were as follows: Group 1 (AVVC), patients with an initial or sporadically occurring episodes of symptomatic vulvovaginitis, that is, occurring fewer than four times a year ($n = 39$ and $n = 30$, respectively); Group 2 (RVVC), symptomatic patients with a history of four or more clinical episodes of VVC per year ($n = 67$ and $n = 64$, respectively); and Group 3 (controls), women who incidentally carried *Candida* in their vagina but were completely asymptomatic and without a history of RVVC ($n = 4$ and $n = 12$, respectively) [1, 5, 27].

The *C. albicans* isolates were recovered on CHROMagar Candida (Becton–Dickinson, Heidelberg, Germany) and identified by their assimilation patterns using the API 20C AUX method (bioMérieux, Marcy l'Étoile, France). We also had previously verified the isolates by using *HWP1* polymorphisms, which yielded a DNA fragment of ~1,000 bp that was identical to that of the reference *C. albicans* strain, ATCC 90028 [28]. For DNA isolation, yeast cells were grown in Sabouraud broth medium at 30 °C.

Microsatellite PCR Amplification

Genomic DNA was extracted from yeast isolates as described previously [29], and 5 µl of DNA was used as a template for PCR amplification. PCR was conducted using labeled fluorochrome primers (*CEF3*-Forward 5′-Hex-TTTCCTCTTCCTTTCATATAGAA-3′ and *CEF3*-Reverse 5′-GGATTCACTAGCAGCAGACA-3′). PCRs were performed in 25 µl reaction volumes containing 1 × PCR buffer (20 mM of Tris HCl, pH 8.4, 50 mM of KCl), 0.5 mM of each dNTP (Fermentas, MA, USA), 0.5 mM of each primer (HEX labeled for *CEF*), 1.5 mM of $MgCI_2$ (Fermentas), 3 µl of genomic DNA, and 1.25 U of Taq DNA polymerase (New England BioLabs, Herts, UK). PCR amplifications were performed in a thermocycler (BOECO, TC-Pro, Germany) operated with a temperature cycling program that consisted of an initial denaturing step at 95 °C for 5 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C, and 45 s at 72 °C. The final extension step was for 5 min at 72 °C. *C. albicans* ATCC 18804 (synonym CBS 562) was used as a positive control to test the accuracy of the typing assay.

Fragment Size Determination

The PCR products were run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) together with the GeneScan-500 (ROX) size standard (Applied Biosystems). Fragment sizes were determined using GeneScan 3.7 Analysis software. The discriminatory power (DP) was calculated using Arlequin 3.5.1.3 software (http://cmpg.unibe.ch/software/arlequin3) [30].

Antifungal Susceptibility Testing

The results of antifungal testing for isolates from 216 women were obtained retrospectively from our laboratory records. These findings have been previously studied by our group [27, 31, 32]. Antifungal testing was conducted according to the guidelines reported in the Clinical Laboratory Standards Institute (CLSI) M27-A3 protocol for the interpretation of antifungal susceptibility. The following antifungal agents were tested at the concentrations listed: amphotericin B (AMB, 0.03–16 µg/ml), FLU (0.12–128 µg/ml), ketoconazole (KET, 0.007–8 µg/ml), nystatin (NYS, 0.06–64 µg/ml), 5-fluorocytosine (5-FU, 0.06–64 µg/ml), and boric acid (BA 0.82–419.84 µg/ml). The interpretive breakpoints for minimal inhibitory concentrations (MICs) were as follows: (1) FLU: ≤8 (µg/ml) susceptible (S), MIC between 16 and 32 (µg/ml) susceptible dose-dependent (S-DD), and MIC ≥64 (µg/ml)-resistant (R); (2) AMB, MIC ≤1 (µg/ml) S; and (3) 5-FU, MIC ≤4 (µg/ml) S, MIC between 8 and 16 (µg/ml) intermediate, and MIC ≥32 (µg/ml) R [32, 33]. The MIC value assigned as resistant for KET was ≥16 µg/ml. However, there are no published interpretation criteria for NYS and BA [27]. Antifungal susceptibility results were obtained by determining the MIC distribution as well as the MIC_{50} and MIC_{90} values for each antifungal agent. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as the CLSI-recommended controls [33, 34].

Statistical Analysis

The statistical analysis was performed using the Statistical Package, SPSS, version 19. For the univariate analysis, the distribution of genotypes among women with different isolates and among pregnant and non-pregnant women was tested by the Kruskal–Wallis test. The susceptibility (S, S-DD, and R) of the isolates to antifungal drugs was cross-classified with pregnancy status, species of isolate (AVVC, RVVC, and control), and genotype. Chi-squared tests were used to determine the association between these variables. In addition, when the response to the antifungal drug was binary, that is, either S or S-DD or S or R, a logistical regression model was employed in order to evaluate whether any of the above factors (pregnancy status, species of isolate, and genotype) had any effect on the response to drugs, first separately (univariate analysis) and then jointly (multiple logistic regression).

Results

The MIC results for the control strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were in the acceptable range. In addition, the MIC range as well as the MIC_{50} and MIC_{90} values was calculated for each of the 216 *C. albicans* isolates. These values are listed in Table 1 for the pregnant and non-pregnant groups, including the AVVC, RVVC, and control groups. All of the cases in the group of pregnant patients and the cases of RVVC in the non-pregnant patient group were designated as complicated VVC. All of the isolates were susceptible to AMB. Two of the *C. albicans* isolates were FLU-resistant. However, 26 of the isolates were FLU S-DD; these were predominately found in pregnant cases with RVVC. Additionally, eight of the isolates were KET-resistant. Although all of the isolates were susceptible to 5-FU, 14 were S-DD. We also observed low MIC values for NYS (0.12–64 μg/ml) and BA (0.82–52.48 μg/ml). In general, the MIC values of the *C. albicans* isolates derived from the pregnant and non-pregnant patients, regardless of the severity of their VVC, were not significantly different ($P > 0.05$; Table 1).

We identified 20 allelic genotypes that were characterized by different *CEF3* allelic combinations. The most common allelic-based genotypes were 136–144 (genotype 17, 27.3 %) and 126–135 (genotype 2, 22.7 %; Table 2). However, no major genotypes were found to cause VVC. In addition, no significant correlation was found between any genotype and antifungal resistance ($P > 0.05$). The DP was calculated as 0.8561 ± 0.0145.

Discussion

We investigated 216 vaginal *C. albicans* isolates and identified 20 allelic combination-based genotypes using the microsatellite marker analysis of *CEF3*. We observed that genotype 17 (136–144) and genotype 2 (126–135) accounted for 50 % of the isolates and that these genotypes were found in both complicated and uncomplicated cases (Table 2). In contrast to the Chinese data currently available in the literature [10–13], we did not find any major genotypes causing complicated VCC. In addition, no *C. albicans* genotypes were correlated with any specific antifungal resistance. To the best of our knowledge, this study is the first to reveal allelic combination-based genotypes of *C. albicans* vaginal isolates in our region. This profile would be helpful in understanding the molecular epidemiology of *C. albicans* and provides molecular data to researchers working on the bioinformatics of *C. albicans*.

In the last decade, short tandem repeats (STRs) or microsatellites have been increasingly used as molecular markers for population genetics, strain identification, and characterization [10, 11, 25, 26]. By using two microsatellite markers, *CAI* and *CEF3*, 116 *C. albicans* strains yielded 87 different multi-locus allelic combinations with a high DP of 0.987 [26]. In an elegant study, Fan et al. [10] reported that the majority of the *C. albicans* strains associated with VVC possessed the same or similar *CAI* genotypes. The authors described 26 distinct genotypes from 93 *C. albicans* strains based on a single-strand conformation polymorphism (SSCP) from a PCR-amplified *CAI* microsatellite locus. The data implied that *C. albicans* strains with certain genotypes might be more virulent than other strains that cause VVC and that strain differences may play a significant role in the etiology of *C. albicans*. The finding was later verified by the same group [11, 13].

The dominant *CAI* genotypes of the strains isolated from the vagina have rarely been found in the strains

Table 1 Antifungal susceptibility of vaginal *C. albicans* isolates recovered from pregnant and non-pregnant women with a variety of infection symptoms

Antifungals		Isolates from pregnant women ($n = 110$)			Isolates from non-pregnant women ($n = 106$)		
		AVVC ($n = 39$)	RVVC ($n = 67$)	Controls ($n = 4$)	AVVC ($n = 30$)	RVVC ($n = 64$)	Controls ($n = 12$)
Amphotericin B (μg/ml)	MIC range	0.03–0.25	0.03–0.25	0.03–0.5	0.03–1	0.03–1	0.03–0.5
	MIC_{50}	0.03	0.06	ND	0.12	0.06	0.03
	MIC_{90}	0.12	0.12	ND	0.25	0.12	0.25
R; ≥2 μg/ml	*n* (%)	–	–	–	–	–	–
Fluconazole (μg/ml)	MIC range	0.12–16	0.25–128	0.5–32	0.25–8	0.25–4	0.25–128
	MIC_{50}	2	8	ND	0.5	1	2
	MIC_{90}	8	16	ND	1	2	4
R; ≥64 μg/ml	*n* (%)	–	1 (1.4 %)	–	–	–	1 (8.3 %)
S-DD; 16–32 μg/ml	*n* (%)	7 (17.9 %)	16 (23.8 %)	2 (50 %)	1 (3.3 %)	–	–
Ketoconazole (μg/ml)	MIC range	0.25–32	0.12–16	1–16	0.25–4	0.25–32	0.25–4
	MIC_{50}	8	4	ND	1	4	2
	MIC_{90}	16	8	ND	2	8	4
R; ≥16 μg/ml	*n* (%)	5 (12.8 %)	1 (1.4 %)	–	–	2 (3.1 %)	–
Nystatin (μg/ml)	MIC range	2–8	0.25–16	4–16	0.12–8	0.12–16	0.12–64
	MIC_{50}	4	4	ND	1	4	4
	MIC_{90}	8	8	ND	2	8	8
ND	*n* (%)	–	–	–	–	–	–
5-Fluorocytosine (μg/ml)	MIC range	0.12–8	0.12–4	0.12–8	0.12–16	0.12–16	0.25–8
	MIC_{50}	2	2	ND	0.25	1	2
	MIC_{90}	4	4	ND	4	4	4
R; ≥32 μg/ml	*n* (%)	–	–	–	–	–	–
S-DD; 8–16 μg/ml		1 (2.5 %)	3 (4.4 %)	1 (25 %)	4 (13.3 %)	4 (6.2 %)	1 (8.3 %)
Boric acid (μg/ml)	MIC range	0.82–52.48	0.82–52.48	0.82–13.12	1.64–26.24	0.82–52.48	0.82–6.56
	MIC_{50}	13.12	13.12	ND	6.56	6.56	1.64
	MIC_{90}	26.24	26.24	ND	13.12	13.12	3.28

R resistance, *S-DD* susceptible dose-dependent, *ND* not determined

examined from other sources (e.g., oral, skin, and rectum) [10, 11]. The two most common genotypes (*CAI* 30–45 and *CAI* 32–46) were increased in patients with complicated VVC [10, 12, 13, 16]. These two genotypes might be more virulent and/or more resistant to the commonly used azole drugs [10, 11]. Remarkably, however, *CAI* 32–46 isolates are usually less susceptible to both FLU and ITR than *CAI* 30–45 isolates [10, 16]. In contrast, Antonopoulou et al. [18] did not find any association between azole-resistant isolates and microsatellite genotype clusters among 249 vaginitis isolates of *C. albicans* from Belgium and Greece. In addition, lower MIC levels of fenticonazole than KET, FLU, and ITR were recorded among isolates using both the CLSI M27-A2 and EUCAST methods, which revealed an excellent agreement rate of 97 % [18].

Ge et al. [13] analyzed 199 *C. albicans* isolates from Chinese women and identified 124 sequence types. The majority of the VVC (71.6 %) and balanitis (92.3 %) isolates from China were located in clade 1 of *C. albicans*, but only 40.6 % of the vaginal isolates and 7.8 % of the oral isolates from healthy volunteers were found in the same clade. Interestingly, the authors observed a remarkable difference in the genotype distribution patterns between Chinese and non-Chinese vaginal isolates (the MLST data of vaginal *C. albicans* isolates were available from other

Table 2 Frequencies of *CEF3* allele associations in 216 independent vaginal *C. albicans* isolates

No	Allelic combination-based genotypes	Isolates from pregnant women			Isolates from non-pregnant women		
		AVVC ($n = 39$)	RVVC ($n = 67$)	Controls ($n = 4$)	AVVC ($n = 30$)	RVVC ($n = 64$)	Controls ($n = 12$)
1	126–126	–	–	–	2	–	–
2	126–135[a]	7	14	–	8	19	1
3	129–156	1	–	–	–	2	–
4	130–130	–	1	–	1	1	–
5	130–135	1	2	–	–	1	–
6	130–136	1	2	–	–	–	1
7	130–144	1	3	–	1	8	1
8	131–131	–	1	–	1	–	–
9	131–139	2	2	–	–	–	–
10	133–136	1	–	–	4	1	2
11	133–144	2	4	–	1	6	–
12	135–135	1	2	–	–	3	–
13	135–139	2	3	1	–	–	–
14	135–152	–	1	–	–	–	–
15	136–135	–	–	1	–	–	–
16	136–136	1	1	1	4	6	1
17	136–144	14	24	1	4	10	6
18	136–145	2	4	–	3	4	–
19	136–146	2	3	–	1	3	
20	145–145	1	–	–	–	–	–

DP (Discriminatory power): 0.856 ± 0.0145

[a] ATCC 18804 = CBS 562 was assigned for this genotype

countries in the MLST database). During 2000–2010, Marchaim et al. [35] identified 25 women with FLU-resistant recurrent *C. albicans* vaginitis who had consumed FLU in the previous 6 months. The authors suggested that the management of FLU refractory VVC is extremely difficult with limited options and that new therapeutic modalities are needed.

In this study, there is little evidence of drug resistance in any of the groups. In addition, there was no correlation between drug susceptibility and genotype. Most of the *C. albicans* isolates exhibited high levels of susceptibility to the antifungals tested, while a small number of the strains exhibited high MICs. Consistent with other reports [15, 16, 18], we observed lower rates for FLU resistance (0.9 %) and a slightly higher rate for S-DD (12 %). Additionally, all of the isolates were susceptible to AMB, and only 3.7 % of the isolates were found to be KET-resistant. Moreover, because this study included isolates from only our hospital, the sample size was too small to identify more FLU- and KET-resistant isolates (Table 1). For 5-FU, resistance and S-DD rates were found to be 0 and 6.5 %, respectively.

In summary, the *CEF3* microsatellite method is reliable and sufficiently discriminatory for differentiating *C. albicans* genotypes. Although two genotypes, 136–144 and 126–135, were present in 50 % of the isolates, we identified 20 allelic combination-based genotypes in total. However, we did not find any specific correlation between genotype, severity of VVC, and antifungal resistance (Table 2; $P > 0.05$). This may suggest that the link between genotype and severity of VVC is peculiar to China [10–13], a possibility which should certainly stimulate further studies by other groups in other regions of the world. An increased focus on the molecular epidemiology of VVC will ultimately lead to better measures for its control.

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