Mycopathologia (2017) 182:339–347
DOI 10.1007/s11046-016-0095-3

Genotyping and Persistence of *Candida albicans* from Pregnant Women with Vulvovaginal Candidiasis

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Received: 8 June 2016 / Accepted: 16 November 2016 / Published online: 23 November 2016

Abstract
Objective To study *Candida albicans* genotypes using RAPD and their susceptibility to fluconazole in healthy pregnant women and in vulvovaginal candidiasis (VVC) patients after topical treatment with clotrimazole.
Methods Vaginal swabs were collected at $t = 0$ and $t = 1$ (1 month later) in pregnant women (control group, $n = 33$), and before ($t = 0$), at 1 month ($t = 1$) and at 2 months ($t = 2$) after clotrimazole treatment in pregnant women with VVC.
Results *Candida albicans* was isolated in 30% of healthy pregnant women and 80% of patients with VVC. A high genetic heterogeneity was observed in *C. albicans* genotypes between individuals. In patients with VVC, topical antifungal treatment with clotrimazole was clinically effective, but only in a 62% *C. albicans* was eradicated. In patients in which *C. albicans* was not eradicated, this microorganism persisted for 1 or 2 months after the antifungal treatment. The persistent colonies were not associated with a specific genotype, but they were associated with

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higher MICs in comparison with colonies isolated from the control group.

Conclusions Therapy with topical clotrimazole, despite a good clinical outcome, could not eradicate completely *C. albicans* allowing the persistence of genotypes, with higher MICs to fluconazole. More studies with higher number of patients are needed to validate this preliminary finding.

Keywords Genotypes · Persistence · RAPD · Resistance · *C. albicans* · Vulvovaginal candidiasis · Clotrimazole · Pregnant women

Introduction

Vulvovaginal candidiasis (VVC) is a common infection caused by *Candida* species. It can affect at least to a 75% of all women during their lives. This infection can be uncomplicated (e.g., sporadic VVC) or complicated (e.g., recurrent VVC), depending on the frequency and severity of the infection [1]. The most prevalent agent is *C. albicans*, but an increasing number of non*albicans* species have been involved in VVC showing higher resistance to antifungal drugs (e.g., *C. glabrata*) [2].

Although *C. albicans* has a clonal origin, the coexistence of genotypic variants among the commensal or during infection state has been reported [3–5]. The origin of these variants within the population can be explained by microevolution, which could predict the appearance of genetic variants with novel phenotypes such as antifungal resistance and improved virulence, thus leading to genetic diversity [3, 4, 6, 7].

Despite the fact *C. albicans* isolates from vaginal discharge are susceptible to fluconazole, resistant strains have been reported that might be associated with a therapeutic failure and/or recurrent VVC [1, 8, 9]. Recurrent VVC, defined as the presence of at least four episodes during a period of 12 months, affects approximately 5–8% of women worldwide [10]. Recurrence could be explained in part by: (1) reinfection caused by *C. albicans* from endogenous microbiota of the intestine, genital tracts, and/or from the sexual partner and (2) an incomplete eradication of *Candida* from the vagina [6, 7].

Azoles are the first-line treatment for candidiasis that inhibits the ergosterol synthesis in the fungal plasmatic membrane. However, these drugs are generally fungistatic, which favor the emergence of strains with secondary drug resistance [11]. The phenomenon of resistance is an evolutionary process that occurs in fungi when they are exposed to an antifungal drug and depends on the existence of genetic variability in the population [12, 13].

In pregnancy, a higher prevalence of *Candida* species is observed compared to nonpregnant women (30 vs 20%, respectively) [14]. This could be due to immunological changes, increased estrogen levels, and a higher vaginal glycogen production during the pregnancy. Some studies have demonstrated an association between vulvovaginal candidiasis and pregnancy complications such as membrane rupture and poor pregnancy outcome. *Candida* eradication reduces the risk of preterm birth. Most of physicians prescribe topical azoles (imidazole, clotrimazole, or miconazole) vaginally to minimize the systemic exposure to drugs in pregnancy [14]. This route is preferable to the oral route mainly in the first trimester of pregnancy. In Chile, the most used topical azole is clotrimazole [15].

In spite of efficacy studies, no information is available about topical antifungal tissue penetration and *Candida* eradication [16]. In addition, according to the fungistatic nature of azoles, some *C. albicans* genotypes may persist after the treatment, particularly if topical antifungals are prescribed, like in pregnancy. In this study, we studied the *C. albicans* genotypes using randomly amplified polymorphic DNA (RAPD) and their susceptibility to fluconazole in persistent and nonpersistent genotypes of *C. albicans* after treatment with clotrimazole.

Materials and Methods

Patients and Controls

Thirty pregnant women with suspected VVC and 33 controls with a gestational age between 6 and 28 weeks were selected at the Gynecological and Obstetric Department of the Clinic Hospital of the University of Chile. All signed an informed consent, and age, weight, pregnancy week, sexual activity, prior VVC and prior antifungal therapy, and vaginal pH were registered (Table 1). Vaginal swabs were collected and transported to the laboratory in Stuart

medium. Patients were treated with vaginal suppositories of clotrimazole, 100 mg/day by 7 days. Sample from VVC patients were collected prior the treatment ($t = 0$), at 1 month ($t = 1$), and at 2 months ($t = 2$) after. For the control group, two samples were collected with 1 month of difference ($t = 0$ and $t = 1$). The Ethical Council from the University of Chile approved this work. Controls and cases, in which *C. albicans* was isolated at least in two moments, were selected for study.

Strains

The samples were streaked on Sabouraud agar and incubated 24–48 h at 37 °C. Each colony of *C. albicans* was identified by conventional and commercial methods (CHROMagar Candida and API ID 32C). The first 30 colonies of *C. albicans* were selected for a susceptibility and genotyping study. Colonies were stored at −80 °C until it use.

Genotyping Analysis

The genetic variability of the isolated colonies in each period was evaluated through random amplified polymorphic DNA (RAPD). The DNA was extracted from the stored colonies using the Wizard Genomic DNA Purification Kit™ (Promega) according the manufacturer's recommendations. The colonies were genotyped by RAPD using three standardized primers OPBA10 (TTCCCCACCC), OPBA06 (GGACGACCGT), and OPBA01 (GGACGTTGAG). Each RAPD reaction was composed by 100 pg of genomic DNA, Taq pol buffer 1X (Invitrogen™), nucleotides dATP, dTTP, dCTP y dGTP (Gibco BRL™) 100 μM each one, $MgCl_2$ 2 mM, primers 10 pmol y Taq polimerase (Invitrogen™) 1U, in a total volume of 25 μL. Polymerase chain reactions (PCRs) were conducted in Eppendorf™ Mastercycler™ Gradient thermal cycler and PX2 thermal cycler (Thermo™). The program included a preheat temperature of 95 °C by 3 min, 40 denaturation cycles at 94 °C by 1 min, an annealing temperature of 35 °C by 2 min, an extension at 72 °C by 2 min, and a final extension at 72 °C by 5 min. The profiles obtained from the colonies of each patient in different sampling periods were analyzed using TREECON version 1.3. The genetic similarities were evaluated by the Dice coefficient [17]. Results were represented in a dendrogram through clustering analysis using the unweight pair group method with arithmetic averages (UPGMA) algorithm (Fig. 1) [18]. Strains were considered highly genetically related if their similarity (S_{AB} value) was $\geq$0.9 and not related if their S_{AB} value was $\leq$0.8 [19].

Table 1 Clinical parameters in patients with suspect of vulvovaginal candidiasis (VVC) and controls

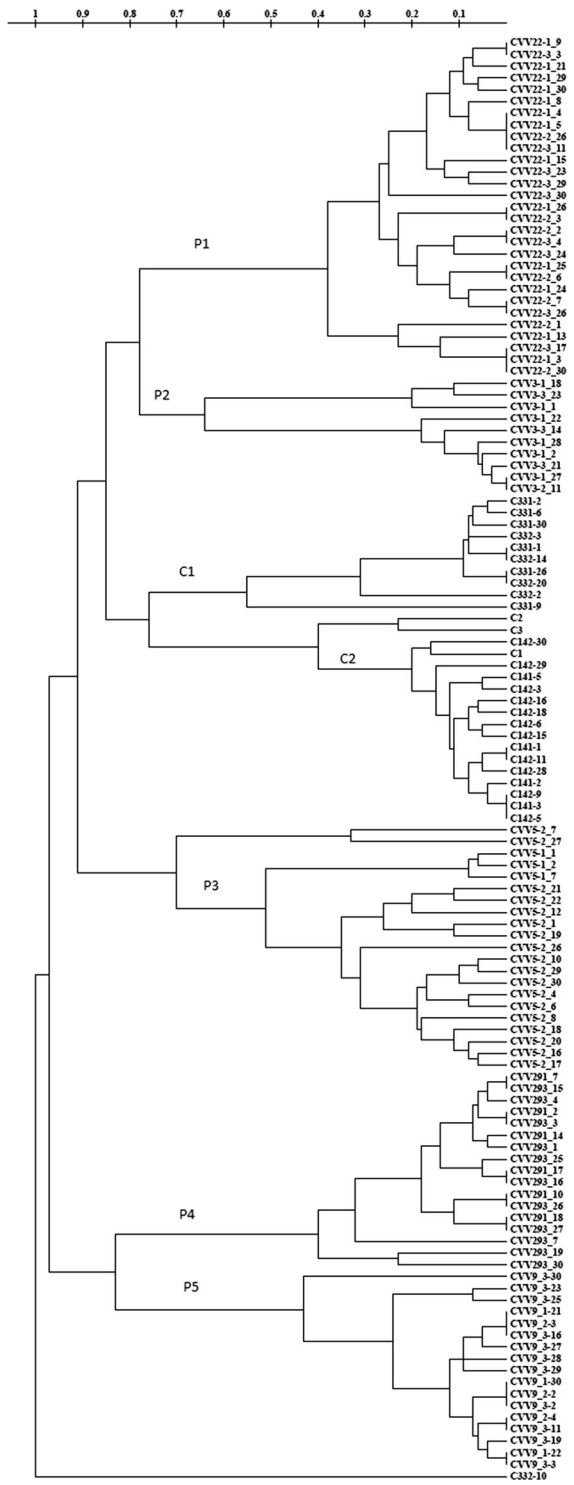
Parameters	VVC $n = 30$	Controls $n = 33$
Vaginal pH	4.5	5.0
Symptoms and signs (%)	100	0
Age (year)	20–43	18–43
Weigh (Kg)	49.9–100	45.5–95.7
Pregnancy week	6–28	8–31
Sexual activity (%)	94.4	93.7
Previous VVC (%)	61.1	63.3
Previous antifungal therapy (%)	50	50

Antifungal Susceptibility Testing

The susceptibility of *C. albicans* to clotrimazole was estimated by measuring the minimal inhibitory concentration (MIC) to fluconazole, given the crossed sensitivity to azole drugs in *Candida*, and there are established breakpoints to fluconazole but no for clotrimazole [20, 21]. The antifungal susceptibility testing to fluconazole was performed according the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (EUCAST, discussion document 7.1) [21].

Statistics

The fluconazole MIC values of nonpersistent and persistent genotypes were expressed as geometric means. As MIC values are noncontinuous variables, there were converted to $\log_2$ MIC (mg/L) values to allow an overall description of the distribution of MIC values. The one-way ANOVA test was applied to compare $\log_2$ fluconazole MICS between controls and vulvovaginal candidiasis cases. The difference of $\log_2$ of fluconazole MICs in persistent and nonpersistent



◀**Fig. 1** Dendrogram including all genotypes ($n = 99$) from controls (C) and vulvovaginal candidiasis (VVC). The values below the *horizontal line* represent the distance between individuals. *C1* control C33, *C2* control C14, *P1* patient VVC22, *P2* patient VVC3, *P3* patient VVC5, *P4* patient VVC29, *P5* patient VVC9

genotypes was tested using the test of student. All statistics were calculated using the GraphPad Prism 6 Software Inc.

Results

Prevalence of *Candida* Species

Among the suspected cases, 21 of them were microbiologically confirmed as VVC, and the isolated species were: 80% *C. albicans* (17/21); 10% *C. glabrata* (2/21); 5% *C. lusitanie* (1/21); and 5% *Candida* sp. (1/21). In comparison, among the 30% of colonized pregnant women (control group), 90% of the isolates were *C. albicans* (9/10) and 10% were *C. kusei* (1/10).

The prevalence of *C albicans* at different times ($t = 0$, $t = 1$ and $t = 2$) is shown in Table 2. All the positive and negative samples were confirmed by using a commercial PCR kit (Bioscan^{TM}, Chile).

In the VVC group, after treatment, the prevalence of *C. albicans* decreased from 80% at $t = 0$ to 27 and 34% at $t = 1$ and $t = 2$, respectively. Treatment with clotrimazole was effective in 62% of patients with microbiologically confirmed VVC at $t = 1$ (13/21) and 52% at $t = 2$ (11/21). Persistence of *Candida* sp. was found in two controls and eight cases. From them, *C. albicans* was isolated in two controls and five VVC patients.

Genetic Diversity

A total of 445 colonies of *C. albicans* from pregnant women, were isolated and genotyped by using RAPD: for the control group, 36 at $t = 0$ and 53 at $t = 1$; for the VVC group, 147 at $t = 0$; 90 at $t = 1$; and 119 at $t = 2$ (Table 3). The genotyping identified 99 distinct *C. albicans* genotypes. Highly related genotypes were identified for each pregnant woman that were grouped in clusters (Fig. 1). For controls, a total of 12 genotypes at $t = 0$ and 29 at $t = 1$ were found, while 65 at $t = 0$, 47 at $t = 1$ and 52 at $t = 2$ were found for VVC cases. The number of persistent genotypes is shown in Table 3. Among genotypes identified in the control group, six genotypes were present in both

Table 2 Positive samples to *Candida* spp. and *C. albicans* in controls and vulvovaginal candidiasis (VVC) cases

	Healthy pregnant women (controls)		Pregnant women with VVC (cases)		
Number of subjects	33	31	30	30	29
Sampling time	$t = 0$	$t = 1$	$t = 0$	$t = 1$	$t = 2$
Positive samples to *Candida* spp.	10/33 (30%)	2/31 (6%)	21/30 (70%)	8/30 (27%)	10/29 (34%)
Positive samples to *C. albicans*	9/10 (90%)	2/2 (100%)	17/21 (80%)	5/8 (63%)	6/10 (60%)

t = 0, initial sampling time, *t = 1*, 1 month after the initial sampling time, *t = 2*, 2 months after the initial sampling time

Table 3 Colonies and genotype number of *C. albicans* isolated from controls (C) and vulvovaginal candidiasis patients (VVC)

	C $t = 0$	C $t = 1$	VVC $t = 0$	VVC $t = 1$	VVC $t = 2$
C. albicans colonies number	36	53	147	90	119
Genotypes by sampling time	12	29	65	47	52
Persistent genotypes	6		11		

t = 0, initial sampling time, *t = 1*, 1 month after the initial sampling time, *t = 2*, 2 months after the initial sampling time

sampling time (G1, G4, G5, G14, G19, and G23) (data not shown). In pregnant women with VVC, 36 different genotypes for *C. albicans* were identified at $t = 0$, genetically distinct from the control group. After the treatment, 11 genotypes persisted either 1 and 2 months after the treatment (G33, G35, G53, G56, G79, G82, and G84) or just 1 month after the treatment (G25, G43, G46, and G74) (data not shown). Among the 23 genotypes not recovered 1 month after the antifungal treatment, eight genotypes reappeared 2 months after the treatment (G27, G55, G92, G94, G95, G98, G100, and G101) (data not shown). Nevertheless, 41 *C. albicans* genotypes after the treatment ($t = 1$ and/or $t = 2$) were not present before the treatment at $t = 0$ (data not shown). The most frequently isolated genotypes (>7%) in VVC pregnant women were the genotypes G33, G35, G40, G55, G56, G58, G79 and G100 (data not shown).

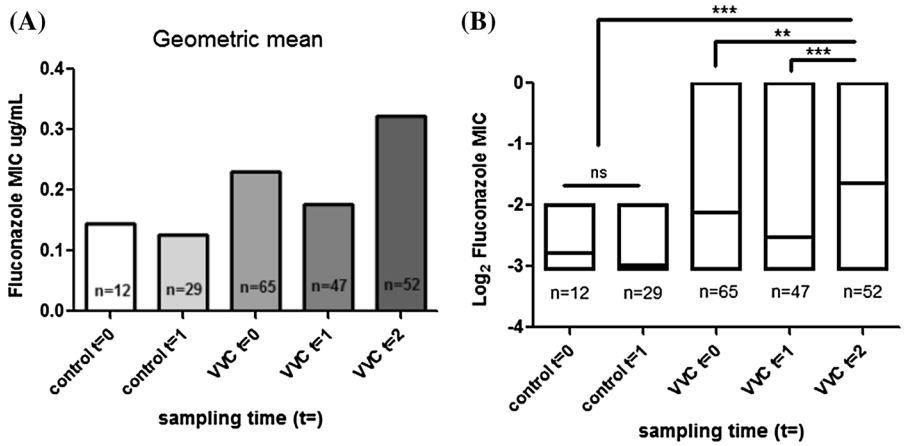


Fig. 2 **a** Fluconazole minimal inhibitory concentrations (MICs) of *C. albicans* total genotypes isolated from controls and vulvovaginal candidiasis (VVC) patients. Columns represent the geometric mean. **b** Logarithm in base 2 ($\log_2$) of minimal inhibitory concentrations (MICs) of *C. albicans* genotypes from controls and vulvovaginal candidiasis (VVC) patients. MICs to fluconazole genotypes isolated from VVC treated woman exhibited higher logarithms in base 2 ($\log_2$) of MICs to fluconazole compared to controls. One-way ANOVA test was applied to compare $\log_2$ fluconazole MICS between controls and vulvovaginal candidiasis cases, and the Bonferronís multiple comparison posttest was applied to study differences between columns. ***$p < 0.0001$; ** $p < 0.01$

Antifungal Susceptibility to Fluconazole

All the isolated colonies from pregnant women were tested in their antifungal susceptibility to fluconazole. As is shown in Fig. 2, genotypes isolated from VVC cases had higher MICs than controls (Fig. 2a) and statistically confirmed by the comparison of the $\log_2$ fluconazole MICs ($p < 0.0001$) (Fig. 2b). On the other hand, between genotypes isolated from VVC cases, those isolated at $t = 2$ showed higher MICs than genotypes isolated at $t = 0$ and $t = 1$ (Fig. 2a) which was corroborated by $\log_2$ fluconazole MICs comparison ($p < 0.01$ and $p < 0.0001$, respectively).

Persistent genotypes showed higher geometric mean of fluconazole MICs (Table 4; Fig. 3a); however, there were no statistical differences in $\log_2$ fluconazole MICs between nonpersistent and persistent genotypes (Fig. 3b). Figure 3c shows MICs of persistent genotypes during sampling time showing a plane regression line, while in VVC cases there is a positive correlation between MICs and sampling time in VVC cases (Fig. 3d).

Discussion

In the present study, we investigated the genetic diversity and persistence of *C. albicans* genotypes in pregnant women with and without vulvovaginal candidiasis and their antifungal susceptibility to fluconazole after topical treatment with clotrimazole. As previously described, *C. albicans* is an opportunistic pathogen associated with vulvovaginal candidiasis in pregnant women, but also is present in the commensal microbiota of control pregnant women group. The topical antifungal treatment with clotrimazole was clinically effective but *C. albicans* was partially eradicated, and persistent genotypes were yet observed patient with VVC 1 and/or 2 months after the treatment.

Here, *C. albicans* vaginal colonization of healthy pregnant women was 30% in agreement with the reported frequencies in the vaginal microbiota of women: for example, *Candida* spp. was retrieved in 37% of asymptomatic Australian women aged <50 years old. In general, 20–30% of healthy women are colonized by *Candida*, which is higher during pregnancy [22–25].

Candida albicans colonies were analyzed using RAPD method. Although several others techniques can be used such as isoenzyme analyses, electrophoretic karyotyping, restriction fragment length polymorphism (RFLP) analyses, and multilocus sequence typing (MLST) to assess genetic diversity of *C. albicans* [4, 26], RAPD is a relatively cost-effective technique with good discrimination potential for *C. albicans* [4, 26–28] and/or other *Candida* species [7, 29–32].

In this work, *C. albicans* strains isolated from pregnant women with VVC were genetically different from the control group and no genotypes in particular

Table 4 Minimal inhibitory concentrations (MICs) of persistent genotypes of *C. albicans* isolated from controls and vulvovaginal candidiasis patients (VVC)

Column statistics	Controls (μg/mL)		VVC patients (μg/mL)		
	$t = 0$	$t = 1$	$t = 0$	$t = 1$	$t = 2$
	0.12	0.12	0.12	0.5	0.5
	0.25	0.25	0.25	1	1
	0.12	0.12	0.12	0.12	0.12
	0.12	0.25	0.25	0.12	1
	0.25	0.12	0.12	0.25	0.5
	0.12	0.12	0.12	0.25	0.12
	0.12	0.12	0.12	0.12	0.25
		0.12	0.12	0.5	0.25
		0.12	0.12	0.12	0.12
		0.12	0.12	0.25	0.5
		0.12	0.12	0.25	0.25
		0.12	0.12	0.5	0.5
			0.25	0.12	0.5
			0.25	0.12	0.5
			0.5	0.25	0.25
			0.25	0.25	1
			0.5		0.25
			0.5		0.25
			0.5		0.25
			0.5		
			1		
			0.25		
			0.12		
			0.12		
n	7	12	24	16	19
Mean	0.16	0.14	0.27	0.30	0.47
SEM	0.02	0.01	0.04	0.06	0.07
Median	0.12	0.12	0.19	0.25	0.25
Geometric mean	0.12	0.12	0.19	0.25	0.25

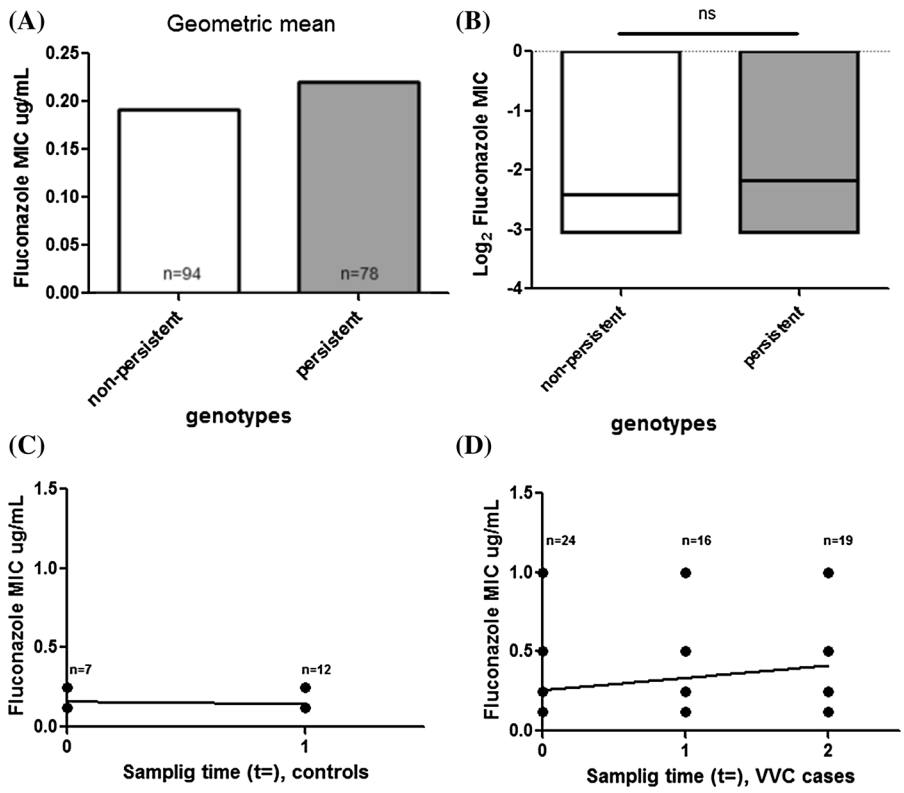


Fig. 3 **a** Fluconazole susceptibility of nonpersistent and persistent genotypes from controls and cases. Columns represent geometric mean. **b** Logarithm in base 2 ($\log_2$) of minimal inhibitory concentrations (MICs) MICs to fluconazole calculated for nonpersistent and persistent genotypes. No statistical differences were found between both groups ($p = 0.1248$ by student t test). **c** Sampling time versus MICs to fluconazole in persistent genotypes isolated from controls and **D** vulvovaginal candidiasis (VVC) patients. Controls: $r^2 = 0.01984$, slope deviation from zero not significant. VVC cases: $r^2 = 0.06933$, slope deviation from zero significant $p = 0.0439$

were associated with VVC. We observed that each related genotypes of *C. albicans* strains were isolated from a unique pregnant woman (Fig. 1). In spite of the fact that *C. albicans* has a clonal origin, the coexistence of genotypic variants in the commensal or infection state have been reported [3–5]. The origin of these variants within the population can be explained by microevolution. Microevolution would predict the appearance of genetic variants with new phenotypes, such as the resistance to antifungal drugs, improved virulence, and moreover, it would increase the genetic diversity of the population [3, 4, 6, 7].

Previously, high genotype heterogeneity has been also observed among oral *C. albicans* strains isolated from patients with cancer receiving chemotherapy in China [27]. This high genotype heterogeneity showed that the host plays an important role on the genotype determination of *C. albicans* colonizer strains. Indeed, the host may be implicated in the transformation of *Candida* from a commensal to a pathogenic organism since a large percentage of colonized women have not symptoms. However, VVC would be caused by host factors such as deficient adaptive immune response, and it has been also shown that it would be caused by an aggressive innate immune response [1, 33]. So, not only the *C. albicans* genotype but also host factors will be implicated in the determination of some pathogenic phenotype but also host factors.

All pregnant women with VVC were clinically cured after the antifungal treatment; however, a part of them remained still colonized by *C. albicans*. To understand this point, we determined the minimal inhibitory concentration (MIC) of the *C. albicans* colonies isolated in cases in whom persistence was observed. Our study revealed that the minimal inhibitory concentration (MIC) to fluconazole was a determinant factor for *C. albicans* persistence (Fig. 3). Although two controls and five cases were eligible in this study, which is a limiting factor because the low number of studied individuals, we analyzed a high number of colonies. In effect *C. albicans* genotypes associated with VVC had higher MICs to fluconazole previous and post treatment with topical clotrimazole than strains isolated from controls, especially at $t = 2$ (Fig. 2), showing MIC values higher than 0.5 µg/mL. In addition, 30% of pregnant women treated with the antifungal were still colonized by *C. albicans* after the treatment by new and persistent genotypes. Also, a

positive correlation between fluconazole MICs and sampling time after treatment was demonstrated (Fig. 3d). The presence of persistent genotypes would explain recurrence associated to higher MICs. During the last years, the concept of epidemiologic cutoff values (ECVs) has been incorporated to assess the emergence of nonwild-type strains, which have a reduced susceptibility to antifungals. While clinical break points (CBPs) allow detecting strains that probably will respond to antifungal treatment, ECVs means the upper limit of MIC distributions that are established for each *Candida* species and can indicate future acquisition of high-level clinically resistance. In the case of *C. albicans*, a susceptible strain must have MIC $\leq$2 μg/mL to fluconazole to assure the success of the treatment, and a MIC <0.5 μg/mL reveal a sensible wild-type strain. In this study there were genotypes with MICs of between 0.5 and 1 μg/mL in VVC cases, especially after treatment. That means that although those genotypes are clinically susceptible, they have MICs over the ECVs indicating that they are nonwild-type strains and future risks to acquire high-level of resistance exist [34, 35]. Nevertheless, studies are needed to explain how antifungal resistance would give a growth advantage for *C. albicans* strains.

To conclude, a high genetic heterogeneity among *C. albicans* genotype isolated in pregnant women was observed between individuals. For pregnant women with VVC, the topical antifungal treatment with clotrimazole was clinically effective but partially eradicated *C. albicans*, persisting for 1 or 2 months after the antifungal treatment. Interestingly, our work shows that the persistence of *C. albicans* colonies is associated with a higher MICs, and this may explain the recurrent infections observed in some pregnant women with VVC, but this point needs more studies in the future to be confirmed.

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