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Yeast vaginitis during pregnancy: susceptibility testing of 13 antifungal drugs and boric acid and the detection of four virulence factors

AYŞE KALKANCI*, AHMET BARIŞ GÜZEL†, ISRAA IBRAHIM JABBAN KHALIL*, MERVE AYDIN*, MACIT ILKIT‡ & SEMRA KUŞTIMUR*

**Department of Medical Microbiology, Faculty of Medicine, University of Gazi, Ankara, †Department of Obstetrics and Gynecology, Faculty of Medicine, and ‡Division of Mycology, Department of Microbiology, Faculty of Medicine, University of Çukurova, Adana, Turkey*

A higher prevalence of vulvovaginal candidiasis (VVC) is seen in pregnant women compared with those who are not pregnant. Recurrence is also more common in pregnant women, and therapeutic responses are reduced. In this investigation, 207 vaginal yeast isolates recovered from pregnant women were tested for susceptibility to 13 antifungal drugs and boric acid and through these studies four virulence factors were also determined. The isolates were recovered from vaginal samples of patients with acute VVC [AVVC, ($n = 73$)], symptomatic recurrent VVC [RVVC, ($n = 89$)], asymptomatic RVVC ($n = 27$), and those without signs and symptoms ($n = 18$). *Candida albicans* was the most common species found (59.9%), followed by *C. glabrata* (19.8%), other *Candida* spp., (19.8%), and *Saccharomyces cerevisiae* (0.5%). Antifungal susceptibility testing was performed as described in CLSI document M27-A3. Additionally, we examined phospholipase and proteinase production, adhesion to vaginal epithelial cells and hemolytic activity. Notably, the MIC values of *Candida* spp. isolates derived from patients with VVC were no different from those of the controls ($P > 0.05$). In addition, *Candida* isolates derived from patients with AVVC or RVVC produced significantly higher amounts of phospholipase and proteinase compared with the controls ($P < 0.05$). Antifungal testing and the determination of virulence factors may lead to the effective and prompt treatment of VVC, particularly in pregnant women.

Keywords antifungal susceptibility, *Candida albicans*, phospholipase, pregnancy, proteinase, imidazoles

Introduction

Vulvovaginal candidiasis (VVC), particularly the recurrent form (RVVC), is a multifactorial disease (e.g., pregnancy, diabetes mellitus, and use of antibiotics) that affects 75% of women at least once during their lifetime. In additon, approximately 50% of these women will also suffer a single recurrence [1]. The majority of cases can be classified as uncomplicated VVC and respond within a few days to a short course of topical or systemic treatment. However, a small minority of women (5–8%) experience RVVC, which is defined as ≥ 4 episodes per year. Additionally, the treatment of RVVC requires extensive therapies that entail multiple weeks of oral and/or systemic treatment [1–3].

It is believed that the high estrogen levels and high glycogen content in vaginal secretions during pregnancy provide an excellent carbon source and increase a woman's risk of developing VVC [4]. Estrogen also enhances the

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Correspondence: Macit Ilkit, Division of Mycology, Department of Microbiology, Faculty of Medicine, University of Çukurova, Adana, 01330, Turkey. Tel: +90532 286 0099; Fax: +90322 457 3072; E-mail: milkit@cu.edu.tr

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adherence of yeast cells to the vaginal mucosa [1]. In addition, hyphal formation and enzymes such as secreted aspartyl proteinases (SAPs) and phospholipases enhance colonization. Although the presence of symptoms is not always related to the yeast load, VVC is associated with increased concentration of *Candida* cells and hyphal elements [3]. As VVC is so common in women during their childbearing years, it is important to understand the pathology of this disease and the safety or risks of the drugs used to treat it during pregnancy [5,6].

Antifungal agents used for the treatment of VVC include imidazole antifungals [e.g., clotrimazole (CLO), ketoconazole (KET), miconazole (MICO), and tioconazole (TIO)], triazole antifungals [e.g., fluconazole (FLU) and itraconazole (ITR)], and polyene antifungals [e.g., nystatin (NYS)] [7]. The topical formulations of imidazole and triazole antifungals, are considered the therapy of choice during pregnancy based on the safety data collected from studies using animals and humans [8,9]. There are commercially available topical vaginal formulations of CLO, econazole (ECO), KET, MICO, TIO, and NYS. Systemic absorption of these topical medications is minimal, meaning little risk of transfer to the unborn fetus [7].

The U.S. FDA definitions for pregnancy risk of drugs are as follows: (i) Pregnancy Category A, controlled studies in pregnant women fail to demonstrate that a drug is a risk to the fetus in all trimesters; (ii) Pregnancy Category B, risk cannot be ruled out; (iii) Pregnancy Category C, positive evidence of risk, teratogenic or embryocidal effects; (iv) Pregnancy Category D, contraindicated in pregnancy, but may be acceptable despite the risk; and (v) Pregnancy Category E, contraindicated in women who are or may become pregnant [10]. In terms of fetal toxicity, CLO, terbinafine (TER), and amphotericin B (AMB) are classified in Category B. Notably, ECO, KET, MICO, sulconazole (SUL), TIO, FLU, ITR, 5-fluorocytosine (5-FU), and caspofungin (CAS) are Category C agents [10]. Topical NYS is a safe alternative to azoles, and its effects during the first trimester of pregnancy have been extensively studied [7]. The topical vaginal form of NYS is a Category A agent, and the topical form is a Category B agent [10]. Oral FLU is widely used to treat VVC but is considered a second-line drug, as it is a Class C agent when used during pregnancy [9]. Additionally, the fetal toxicity of boric acid (BA) is undetermined [7].

Although antifungal testing provides relatively reliable data that can be used to adjust the management of intensive care patients with invasive *Candida* disease, the testing results do not correlate well with the clinical responses of patients with *Candida* vaginitis, thereby limiting its use for this population [2,11]. However, it is reasonable to perform susceptibility tests for women who have poor clinical and mycological responses during individual episodes or for those receiving maintenance suppressive azole regimens who have breakthrough infections [11]. Fungal culture, antifungal testing, and the examination of virulence factors may be important for the optimal management and the control or eradication of VVC in pregnant women. The present study was designed to achieve the following aims: to determine the *in vitro* antifungal susceptibility and the virulence factors of *Candida* strains derived from pregnant women with AVVC or asymptomatic or symptomatic RVVC as compared with controls.

Materials and methods

Study population

Patients with VVC were experiencing symptoms such as vulvar and vaginal itching, vulvar burning, 'cottage-cheese-like' discharge, dyspareunia, and external dysuria. The following are the definitions of VVC, clinical presentations, and the numbers of isolates studied: Group 1, AVVC, patients with their first or a sporadic episode of symptomatic vulvovaginitis, i.e., less than four times a year, $n = 73$ (35%); Group 2, symptomatic RVVC, symptomatic patients with a history of four or more clinical episodes of VVC per year, $n = 89$ (43%); Group 3, asymptomatic RVVC, asymptomatic patients with a history of RVVC, $n = 27$ (13%); and Group 4, controls, women who incidentally were found to carry significant numbers of *Candida* in the vagina, even though they were completely asymptomatic and without a history of RVVC, $n = 18$ (9%) [2,12].

Yeast isolates

The isolates were derived from pregnant patients aged 18–49 years. Vaginal isolates ($n = 207$) were identified and deposited at the Division of Mycology, Department of Microbiology, Faculty of Medicine, Çukurova University. A subset of these *Candida* isolates ($n = 206$) was the subject of a recent publication by our group [12]. Additionally, the IGS 1 region (approximately 500 bp) of one *Saccharomyces cerevisiae* strain was sequenced using an ABI 3700 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.

Antifungal susceptibility testing

Antifungal testing was conducted at the Department of Medical Microbiology, Faculty of Medicine, Gazi University, Ankara. Criteria derived from the Clinical and Laboratory Standards Institute (CLSI) M27-A3 protocol guided the interpretation of antifungal susceptibility. The following antifungal agents were tested at the concentrations listed: AMB (0.03–16 μg/ml); FLU (0.12–128 μg/ml); ITR

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(0.03–16 μg/ml); KET (0.007–8 μg/ml); NYS (0.06–64 μg/ml); 5-FU (0.06–64 μg/ml); BA (0.82–419.84 μg/ml); CAS (0.03–16 μg/ml); MICO (0.007–8 μg/ml); ECO (0.007–8 μg/ml); TIO (0.06–64 μg/ml); CLO (0.06–64 μg/ml); SUL (0.03–16 μg/ml); and TER (0.06–64 μg/ml).

Briefly, minimal inhibitory concentrations (MICs) were determined for each antifungal agent and used to classify the susceptibility of the isolates as follows: (i) FLU: ≤ 8 (μg/ml) susceptible (S), MIC between 16 and 32 (μg/ml) susceptible dose-dependent (S-DD), MIC ≥ 64 (μg/ml) resistant (R); (ii) ITR, MIC ≤ 0.125 (μg/ml) S, MIC between 0.25 and 0.5 (μg/ml) S-DD, MIC ≥ 1 (μg/ml) R; (iii) AMB, MIC ≤ 1 (μg/ml) S; (iv) 5-FU, MIC ≤ 4 (μg/ml) S, MIC between 8 and 16 (μg/ml) intermediate (I), MIC ≥ 32 (μg/ml) R. However, there are no published interpretation criteria for other antifungal agents. All isolates of *C. krusei* were declared resistant to FLU. Antifungal susceptibility results were demonstrated as the MIC distribution, MIC_{50} and MIC_{90} values for each antifungal agent. *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as CLSI-recommended controls [13,14].

Virulence factors

All of the isolates were tested for adhesion to vaginal epithelial cells (VECs), hemolytic activity, and proteinase and phospholipase production.

Adhesion assay

The adhesion test was performed as described by Biasoli *et al*. [15]. The VECs were obtained from the vaginas of healthy individuals. For each isolate, 500 μl of yeast suspension was mixed with 500 μl of the VEC pool and incubated at 37°C with agitation for 1 h. The mixture was filtered through a 20 μm pore membrane filter. The cells were fixed with methanol and stained using the Gram technique. After staining, the number of cells that adhered to 100 VECs was counted. The assay protocol was reviewed and approved by the Gazi University Ethical Committee (No: 071/2010) for the use of VECs obtained from healthy women.

Determination of hemolytic activity

Hemolytic activity was assayed using a method described by Luo *et al*. [16]. A loopful of the stock culture was streaked onto Sabouraud dextrose agar (SDA) and incubated at 37°C for 18 h. The resultant cultures were harvested and washed with sterile saline, and a yeast suspension with an inoculum size of 10^8 cells/ml was prepared. Ten microliters of this suspension was spot-inoculated on a sugar-enriched sheep blood agar so as to yield a circular inoculation site about 5 mm in diameter. The latter medium was prepared by adding 7 ml of fresh sheep blood (Oxoid, Madrid, Spain) to 100 ml of SDA supplemented with 3% glucose (final concentration, wt/vol). The final pH of the medium so prepared was 5.0–6.0. The plates were incubated at 37°C in 5% CO_2 for 48 h. The presence of a distinct translucent halo around the inoculum site, viewed with transmitted light, indicated positive hemolytic activity. The diameters of the zones of lysis and the colony were measured, and this ratio (equal to or larger than 1) was used as a hemolytic index to represent the intensity of the hemolysin production by different *Candida* species. The assay was conducted in quadruplicate on two separate occasions for each yeast isolate tested. A translucent halo around the inoculum site indicated a positive test for hemolytic activity.

Detection of secreted aspartyl proteinase

Proteinase production was detected using a method described by Rüchel *et al*. [17]. The medium was sterilized by filtration and added to autoclaved 2% agar. Briefly, a single colony (~ 6 mm) of a *Candida* isolate was inoculated onto bovine serum albumin (BSA) agar containing a yeast carbon base (1.17%), protovit (0.25%), and BSA (0.2%), and adjusted to a pH 5.0. The plates were incubated at 37°C for 5 d. Proteolytic activity (Pz) was measured by calculating the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone, as described by Price *et al*. [18]. The halo around the colony produced by proteolytic activity of the *Candida* strain will be referred to as the 'precipitation zone'. *Candida parapsilosis* ATCC 22019 served as a negative control.

Determination of phospholipase activity

Phospholipase production was assayed according to a method described by Price *et al*. [18]. SDA medium (20 ml) supplemented with 1 mol/l sodium chloride, 0.005 mol/l calcium chloride, and 8% sterile egg yolk emulsion was poured onto 90 mm diameter plates. A single colony (~ 6 mm) of each yeast isolate was inoculated onto the surface of the medium. The plates were incubated at 37°C for 5 d. Phospholipase activity (Pz) was measured as described above for the proteinase assay. *Candida albicans* ATCC 10231 served as a positive control.

Statistical analysis

The results based on continuous measurement were analyzed using a one-way analysis of variance (ANOVA). The Bonferroni test was used as a post hoc analysis. A P-value < 0.05 was considered significant. For each

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virulence factor, a 95% confidence interval (CI) on difference was obtained. When the CI included zero, it indicated no difference between study groups, e.g., (i) group 1 + group 2 + group 3 vs. group 4; (ii) group 1 vs. group 2; or (iii) group 2 vs. group 3 at the 0.05 significant level.

Results

The following strains were examined; *C. albicans* (124 isolates), *C. glabrata* (41), *C. tropicalis* (28), and non-*C. albicans Candida* species (13) [i.e., *C. kefyr* (4), *C. krusei* (4), *C. famata* (2), *C. guilliermondii* (1), *C. parapsilosis* (1), and *C. lusitaniae* (1)], and *S. cerevisiae* (1) (Table 1). 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, MIC_{50} and MIC_{90} values were calculated. These values are listed in Table 2 for each according to *Candida* spp. and the AVVC, symptomatic RVVC, asymptomatic RVVC, and control groups. The MIC_{50} and MIC_{90} values of the tested antifungal agents varied slightly among the isolated yeasts, and a few resistant yeasts were detected in the RVVC group.

In this investigation, the three imidazoles (CLO, KET, and MICO) were 90–98% active against the strains. Briefly, one *C. albicans*, two *C. glabrata*, and one *C. guilliermondii* isolates were FLU-resistant. Additionally, a total of 14 *Candida* spp., which exhibited S-DD for FLU, included nine *C. albicans*, two *C. glabrata*, two *C. tropicalis*, and one *C. famata*. Nevertheless, most of the strains recovered from the AVVC and RVVC groups showed susceptibility to FLU. In general, the MIC values of *Candida* spp. isolates derived from patients with RVVC were no different from those of patients with AVVC or the controls ($P > 0.05$). Table 2 summarizes the *in vitro* susceptibilities of the *Candida* spp. to the 13 antifungal agents and BA.

Of the *Candida* strains isolated from the symptomatic RVVC group, 79% displayed at least one of the virulence factors tested, whereas 74% of the strains from the asymptomatic RVVC group were positive for any virulence factor tested. Only 62% ($P < 0.01$) of the strains isolated from the AVVC patients and 50% ($P < 0.01$) of the controls possessed virulence factors (Table 3). Light microscopy was use to evaluate the adherence to VECs of *C. albicans*. From each group, 20 vaginal *C. albicans* isolates (80 isolates in total) were tested for adherence to pooled normal VECs. The following percentages of isolates from the AVVC, symptomatic RVVC, asymptomatic VVC, and control groups exhibited adherence to VECs: 58%, 63%, 59%, and 44%, respectively ($P > 0.05$). Overall, *C. albicans* isolates were more adherent to VECs than non-*C. albicans Candida* isolates ($P < 0.001$). In this investigation, hemolytic activity was detected in isolates from 70 of 73 cases of AVVC (96%), 80 of 89 cases of symptomatic RVVC (90%), 23 of 27 cases of asymptomatic RVVC (85%), and 15 of 18 controls (83%) ($P > 0.05$). The hemolytic activities of *Candida* strains, expressed as the hemolysis index (Hi), varied from 0.55 (having a well-defined hemolysis zone) to 1.0 (the absence of hemolytic activity). In *C. albicans*, the Hi varied from 0.55–0.85.

Proteinase activity was detected in isolates from 60 of 73 (82%) cases of AVVC, 82 of 89 (92%) cases of symptomatic RVVC, 20 of 27 (74%) cases of asymptomatic RVVC, and nine of 18 (50%) controls. There was a significant difference in proteinase production between the isolates from the VVC and control groups ($P < 0.05$). However, there was no significant difference between the AVVC and RVVC groups ($P > 0.05$). Proteinase production was detected in 80% of the *C. albicans* isolates and 52% of the non-*C. albicans Candida* isolates in the AVVC group, 87% of the *C. albicans* isolates and 56% of the non-*C. albicans Candida* isolates in the symptomatic RVVC group, 85% of the *C. albicans* isolates and 52% of the non-*C. albicans Candida* isolates in the asymptomatic RVVC group, and 31% of the *C. albicans* isolates and 25%

Table 1 The distribution of 207 isolates from pregnant women according to the clinical presentation of yeast vaginitis.

Species	AVVC [$n = 73$ (35.3%)]	sRVVC [$n = 89$ (43%)]	asRVVC [$n = 27$ (13%)]	Controls [$n = 18$ (8.7%)]	Total ($n = 207$)
Candida albicans	46	57	14	7	124
C. glabrata	15	14	7	5	41
C. tropicalis	9	12	4	3	28
C. kefyr	–	1	1	2	4
C. krusei	2	2	–	–	4
C. famata	1	1	–	–	2
C. guilliermondii	–	1	–	–	1
C. parapsilosis	–	–	1	–	1
C. lusitaniae	–	–	–	1	1
Saccharomyces cerevisiae	–	1	–	–	1

AVVC, acute vulvovaginal candidiasis; sRVVC, symptomatic recurrent vulvovaginal candidiasis; asRVVC, asymptomatic recurrent vulvovaginal candidiasis.

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Table 2 The MIC ranges for vaginal yeast isolates from pregnant women treated with 13 antifungals and boric acid.

		Candida albicans (n = 124)				*C. glabrata* (n = 14)				Other yeast spp. (n = 42)			
Antifungals		AVVC (n = 46)	sRVVC (n = 57)	asRVVC (n = 14)	Controls (n = 7)	AVVC (n = 15)	sRVVC (n = 14)	asRVVC (n = 7)	Controls (n = 5)	AVVC (n = 12)	sRVVC (n = 18)	asRVVC (n = 6)	Controls (n = 6)
Amphotericin B (μg/ml)	MIC range	0.03–0.12	0.03–0.25	0.03–0.5	0.03–0.5	0.03–0.5	0.03–0.25	0.03–0.06	0.03–0.12	0.03–0.06	0.03–0.12	0.03–0.25	0.03–0.12
	MIC_{50}	0.03	0.06	0.03	–	0.06	0.06	–	–	0.03	0.03	–	–
	MIC_{90}	0.12	0.12	0.06	–	0.25	0.12	–	–	0.06	0.06	–	–
R; ≥ 2 μg/ml	n (%)	–	–	–	–	–	–	–	–	–	–	–	–
Fluconazole (μg/ml)	MIC range	0.12–16	0.5–128	0.5–32	0.5–32	0.25–8	0.25–64	0.25–8	1–128	0.25–16	1–16	1–64	0.5–4
	MIC_{50}	0.5	2	2	–	1	2	–	–	2	1	–	–
	MIC_{90}	2	4	4	–	2	4	–	–	4	2	–	–
R; ≥ 64 μg/ml[a]	n (%)	–	1 (2%)	–	–	–	1 (7%)	–	1 (20%)	–	–	1 (17%)	–
S-DD; 16–32 μg/ml[a]	n (%)	2 (4%)	4 (7%)	2 (14%)	1 (14%)	–	1 (7%)	–	1 (20%)	1 (8%)	1 (5%)	1 (17%)	–
Itraconazole (μg/ml)	MIC range	0.03–1	0.06–0.5	0.03–1	0.06–0.5	0.12–1	0.06–1	0.12–0.5	0.12–1	0.06–1	0.12–0.5	0.12–1	0.12–0.5
	MIC_{50}	0.25	0.25	0.25	–	0.25	0.12	–	–	0.25	0.12	–	–
	MIC_{90}	0.5	0.5	0.5	–	0.5	0.25	–	–	0.5	0.25	–	–
R; ≥ 1 μg/ml	n (%)	2 (4%)	–	1 (7%)	–	1 (7%)	2 (14%)	–	1 (20%)	1 (8%)	–	1 (17%)	–
S-DD; 0.25–0.5 μg/ml	n (%)	6 (13%)	8 (14%)	2 (14%)	1 (14%)	2 (13%)	1 (7%)	2 (28%)	1 (20%)	3 (25%)	1 (5%)	1 (17%)	1 (17%)
Ketoconazole (μg/ml)	MIC range	0.06–16	0.12–16	0.06–16	0.25–8	0.25–16	0.06–8	0.25–16	0.25–16	0.25–4	0.12–4	0.25–8	0.25–16
	MIC_{50}	1	1	0.5	–	0.5	0.12	–	–	0.5	1	–	–
	MIC_{90}	2	2	1	–	1	0.5	–	–	1	2	–	–
R; ≥ 16 μg/ml	n (%)	1 (2%)	1 (2%)	2 (14%)	–	1 (7%)	–	1 (14%)	1 (20%)	–	–	–	1 (17%)
Nystatin (μg/ml)	MIC range	0.06–2	0.5–4	0.25–2	0.25–1	0.5–2	0.25–1	0.12–2	0.06–1	0.25–4	0.25–4	0.5–2	0.25–2
	MIC_{50}	0.5	0.5	0.25	–	0.5	0.5	–	–	0.5	0.5	–	–
	MIC_{90}	1	1	0.5	–	1	1	–	–	1	1	–	–
ND	n (%)	–	–	–	–	–	–	–	–	–	–	–	–
5-Flucytosine (μg/ml)	MIC range	0.12–8	0.12–8	0.25–4	0.12–8	0.12–4	0.12–16	0.12–2	0.25–16	0.25–1	0.12–4	0.25–16	0.12–2
	MIC_{50}	0.25	2	1	–	1	4	–	–	0.5	1	–	–
	MIC_{90}	4	4	2	–	2	8	–	–	1	2	–	–
R; ≥ 32 μg/ml	n (%)	–	–	–	–	–	–	–	–	–	–	–	–
S-DD; 8–16 μg/ml	n (%)	2 (4%)	2 (3%)	–	1 (14%)	–	3 (21%)	–	1 (20%)	–	–	1 (17%)	–
Boric acid (μg/ml)	MIC range	0.82–26.24	1.64–52.48	1.64–26.24	0.82–26.24	1.64–52.48	3.28–52.48	3.28–26.24	1.64–26.24	3.28–26.24	0.82–26.24	1.64–26.24	0.82–13.12
	MIC_{50}	6.56	6.56	6.56	–	13.12	13.12	–	–	6.56	13.12	–	–
	MIC_{90}	13.12	13.12	3.28	–	26.24	26.24	–	–	26.24	26.24	–	–
ND	n (%)												
Caspofungin (μg/ml)	MIC range	0.03–1	0.03–0.12	0.03–0.06	0.03–1	0.03–1	0.03–0.12	0.06–0.12	0.03–1	0.06–1	0.03–0.06	0.03–0.06	0.06–1
	MIC_{50}	0.25	0.03	0.03	–	0.5	0.03	–	–	0.12	0.03	–	–
	MIC_{90}	0.5	0.06	0.03	–	1	0.06	–	–	0.25	0.03	–	–
R; ≥ 2 μg/ml	n (%)	–	–	–	–	–	–	–	–	–	–	–	–
Miconazole (μg/ml)	MIC range	0.01–4	0.007–1	0.12–4	0.25–1	0.06–1	0.25–4	0.06–0.5	0.25–1	0.03–4	0.03–0.25	0.03–0.5	0.25–4
	MIC_{50}	0.06	0.25	0.5	–	0.25	0.12	–	–	0.25	0.06	–	–
	MIC_{90}	1	0.5	1	–	0.5	1	–	–	1	0.12	–	–
R; ≥ 4 μg/ml	n (%)	1 (2%)	–	1 (7%)	–	–	1 (7%)	–	–	1 (8%)	–	–	1 (17%)
Econazole (μg/ml)	MIC range	0.03–2	0.01–2	0.25–2	0.01–2	0.12–2	0.25–2	0.25–2	0.25–0.5	0.01–2	0.06–2	0.06–2	1–2
	MIC_{50}	1	1	0.5	–	1	1	–	–	0.12	0.25	–	–
	MIC_{90}	2	2	1	–	2	2	–	–	0.25	1	–	–
R; ND	n (%)	–	–	–	–	–	–	–	–	–	–	–	–

(Continued)

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Table 2 *(Continued).*

	Candida albicans (n = 124)				*C. glabrata* (n = 14)				Other yeast spp. (n = 42)			
Antifungals	AVVC (n = 46)	sRVVC (n = 57)	asRVVC (n = 14)	Controls (n = 7)	AVVC (n = 15)	sRVVC (n = 14)	asRVVC (n = 7)	Controls (n = 5)	AVVC (n = 12)	sRVVC (n = 18)	asRVVC (n = 6)	Controls (n = 6)
Tioconazole (µg/ml) MIC range	2–8	2–8	2–8	2–4	2–8	2–4	2–8	2–4	1–8	2–8	2–4	2–8
MIC_{50}	2	2	2	–	2	2	–	–	2	2	–	–
MIC_{90}	4	4	4	–	4	4	–	–	4	8	–	–
R; ND n (%)	–	–	–	–	–	–	–	–	–	–	–	–
Clotrimazole (µg/ml) MIC range	0.03–2	0.03–4	0.12–1	0.03–1	0.25–2	0.12–2	0.25–4	0.03–1	0.06–2	0.03–2	0.03–4	0.03–2
MIC_{50}	0.06	0.12	0.12	–	0.03	0.12	–	–	0.12	0.12	–	–
MIC_{90}	0.12	0.25	0.25	–	0.06	0.25	–	–	0.25	0.25	–	–
R; ≥ 0.5 µg/ml n (%)	4 (9%)	3 (5%)	2 (14%)	1 (14%)	2 (13%)	2 (14%)	1 (14%)	1 (20%)	1 (8%)	2 (11%)	1 (17%)	1 (17%)
Sulconazole (µg/ml) MIC range	0.03–2	0.06–4	0.5–4	0.03–4	0.25–4	0.5–4	0.12–2	0.5–2	0.12–2	0.03–4	0.06–4	2–4
MIC_{50}	1	1	1	–	1	2	–	–	0.06	1	–	–
MIC_{90}	2	2	2	–	2	4	–	–	1	2	–	–
R; ND n (%)	–	–	–	–	–	–	–	–	–	–	–	–
Terbinafine (µg/ml) MIC range	0.5–16	0.25–16	0.5–16	2–16	0.5–16	0.5–16	0.5–16	1–16	1–32	0.5–32	2–16	2–16
MIC_{50}	1	0.5	1	–	0.5	1	–	–	1	1	–	–
MIC_{90}	2	1	2	–	1	2	–	–	2	2	–	–
R; ≥ 16 µg/ml n (%)	2 (4%)	2 (4%)	1 (7%)	1 (14%)	1 (7%)	2 (14%)	1 (14%)	1 (20%)	1 (12%)	1 (6%)	1 (17%)	1 (17%)

R, resistance; S-DD, susceptible dose-dependent; ND, not determined; [a]fluconazole was not active against *C. krusei*; AVVC, acute vulvovaginal candidiasis; sRVVC, symptomatic recurrent vulvovaginal candidiasis; asRVVC, asymptomatic recurrent vulvovaginal candidiasis; C, controls.

of the non-*C. albicans Candida* isolates in the control group. The Pz values ranged from 0.20–0.50 for the AVVC group, 0.50–0.80 for the symptomatic RVVC group, 0.55–0.85 for the asymptomatic RVVC group, and 0.10–0.60 for the controls. The mean proteinase activity of isolates from the AVVC (0.250 ± 0.045) and RVVC groups (0.210 ± 0.050) was significantly different from the control groups (0.150 ± 0.020) ($P < 0.05$).

Phospholipase production was detected in isolates from 36 (49%) cases of AVVC, 66 (74%) cases of symptomatic RVVC, 14 (52%) cases of asymptomatic RVVC, and five (28%) controls. There was a significant difference in phospholipase production between the isolates from the VVC and control groups ($P < 0.05$). However, there was no significant difference between the asymptomatic and symptomatic RVVC groups ($P > 0.05$). Phospholipase production was detected in 85% of the *C. albicans* isolates and 51% of the non-*C. albicans Candida* isolates in the AVVC group, 62% of *C. albicans* isolates and 31% of the non-*C. albicans Candida* isolates in the symptomatic RVVC group, 71% of the *C. albicans* isolates and 35% of the non-*C. albicans Candida* isolates in the asymptomatic RVVC group, and 21% of *C. albicans* isolates and 7% of the non-*C. albicans Candida* isolates in the control group. The isolates tested exhibited various degrees of phospholipase activity (Pz value: 1.5–2.5); the greatest phospholipase activity was detected in *C. albicans* isolates.

Discussion

To the best of our knowledge, this study provides the first detailed *in vitro* analysis (testing of antifungal drugs and virulence factors) of pregnant women with *Candida* vaginitis. Several reports have also focused on the antifungal testing of vaginal *Candida* isolates, particularly FLU [11,19–27]. In contrast to oropharyngeal candidiasis, azole resistance remains rare among vaginal isolates. The same is true of patients with RVVC, in which vaginal isolates remain susceptible to azole-based antifungal drugs and do not show increased resistance to any drug despite long-term exposure to azoles [28].

In view of the overall high levels of susceptibility to antifungal agents, a small number of isolates showed high antifungal MICs in this investigation. Briefly, the imidazoles MICO, KET, and CLO were active against 98%, 97%, and 90% of our isolates, respectively. Specific clinical breakpoints for other imidazoles (TIO, ECO, and SUL) have not yet been defined; therefore, their susceptibility profiles cannot be presented as percentages. Gross *et al*. [23] examined 57 vaginal *Candida* spp. (70% were *C. albicans*) recovered from vaginal samples of prostitutes in Costa Rica by the tablet diffusion method (Neo-sensitabs). The majority of isolates

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Table 3 Virulence factors of vaginal yeast isolates.

Virulence factor	Estimate of difference and 95% Confidence Interval			Virulence factor present (%)			
	AVVC + sRVVC + asRVVC vs. C [CI]	AVVC vs. sRVVC [CI]	sRVVC vs. asRVVC [CI]	AVVC ($n=73$) n (%)	sRVVC ($n=89$) n (%)	asRVVC ($n=27$) n (%)	Controls ($n=18$) n (%)
Adhesion to VEC	No [0.16 (−0.11–0.43)]	No [−0.054(−0.22–0.11)]	No [0.037(−0.17–0.26)]	42 (58)	56 (63)	16 (59)	8 (44)
Hemolytic activity	No [0.08 (−0.013–0.29)]	No [0.06(−0.03–0.15)]	No [0.047(−0.125–0.22)]	70 (96)	80 (90)	23 (85)	15 (83)
Proteinase	Yes [0.36 (0.09–0.62)]**	No [−0.099(−0.23–0.017)]	No [0.181(−0.018–0.38)]	60 (82)	82 (92)	20 (74)	9 (50)
Phospholipase	Yes [0.34 (0.095–0.51)]**	Yes [−0.248(−0.41–0.09)]**	No [0.223(−0.01–0.46)]	36 (49)	66 (74)	14 (52)	5 (28)
Any virulence factor	No [0.21 (−0.056–0.485)]	Yes [−0.17(−0.32–0.017)]*	No [0.046(−0.016–0.26)]	45 (62)	70 (79)	20 (74)	9 (50)

VEC, vaginal epithelial cells; AVVC, acute vulvovaginal candidiasis; sRVVC, symptomatic recurrent vulvovaginal candidiasis; asRVVC, asymptomatic recurrent vulvovaginal candidiasis; C, controls; CI, confidence interval; $^{**}P<0.001$; $^{*}P<0.01$.

were susceptible to KET (91%), FLU (96.5%), and ITR (98%). However, the lowest susceptibility was to MICO (63%), with 28% having an intermediate response, and 9% being resistant.

In the literature, there is a consensus that the resistance of vaginal *C. albicans* isolates to FLU is infrequent [11,19,20,22,26]. In one case report, FLU-resistant (MIC ≥ 64 μg/ml) *C. albicans* vaginitis was also reported [29]. In a Turkish study of vaginitis, 1 of 21 *C. albicans* and 1 of 4 *C. glabrata* isolates were found to be resistant to FLU (≥ 64 μg/ml) by the CLSI M27-A2 method [24]. Recently, Fan and Liu [26], using agar diffusion or E-test methods, reported that FLU-resistance and S-DD rates of 132 vaginal *Candida* isolates were 0.8% and 5.3%, respectively. In this current study, we observed slightly higher rates for FLU as 1.9% and 6.8% by using the CLSI M27-A3 method, respectively.

In one recent study, despite the widespread use of FLU as a treatment for uncomplicated and complicated *C. albicans* vaginitis, no clinically significant increases in the MIC_{50} or MIC_{90} were noted from 1986–2008 by using the CLSI M27-A method. During the same period, when isolates were tested against CLO, MICO, KET, VOR, and AMB, there were similarly no clinically significant changes seen in the susceptibility trends [25]. Notably, *C. albicans* is more susceptible to azoles, but non-*C. albicans Candida* species are more resistant, possibly due to the use of over-the-counter azole agents [22]. Therefore, the use of NYS for the treatment of VVC caused by either azole-susceptible or azole-resistant *Candida* is strongly recommended [26]. In another study, Otero *et al*. [21] used the CLSI M27-A method to test 106 *C. albicans* and 23 *C. glabrata* isolates recovered from prostitutes with vaginitis. They found that all the yeast isolates were inhibited by ≤ 1 μg/ml of AMB; however, 2 of 108 (1.8%) isolates of *C. albicans* were found to be resistant to 5-FU. In addition, *C. albicans* exhibited greater susceptibility to FLU, with MIC_{50} and MIC_{90} of 0.5 μg/ml. However, 4 of 23 (17.3%) *C. glabrata* strains were S-DD (a MIC of 16–32 μg/ml) and 1 strain (4.3%) was found to be resistant (MIC ≥ 64 μg/ml).

Ribeiro *et al*. [20] studied 80 vaginal isolates and reported that ITR exhibited high *in vitro* activity in 53 of 56 *C. albicans* isolates and 28 of 34 non-*C. albicans Candida* isolates by the CLSI M27-A method. For KET, a total of 8 S-DD isolates (two *C. albicans* and six non-*C. albicans Candida* spp.) have been reported. In addition, all isolates were reported as susceptible to AMB. In one study, however, the reported MIC ranges for ITR (< 0.03–2 μg/ml) and AMB (< 0.03–0.5 μg/ml) were narrow; this was also measured using the CLSI M27-A2 method [24]. In this present study, the highest MIC value for AMB among all *Candida* isolates tested was 0.5 μg/ml. In addition, *Candida* spp. MIC_{50} and MIC_{90} values were 0.5–1 and 1–2 μg/ml, respectively, for TER, which is not widely used for VVC (Table 2).

BA, an antiseptic, has been known for at least three decades to be effective for the empirical treatment of VVC [30]. However, there are few data regarding its *in vitro* antifungal activity [21,31]. Using the agar dilution method, *C. albicans* isolates were inhibited by 0.3% BA at rates of 62–66%, and by 0.4% BA at rates of 97.2–100% [21,31]; 91% of *C. glabrata* isolates were inhibited by ≥ 0.6% BA [21]. In addition, we observed extremely low MIC values for BA (≤ 52.48μg/ml; Table 2). Richter *et al*. [22] noted that, among patients with recurrent positive cultures, 34 of 70 (49%) of evaluable episodes of *C. glabrata* vaginitis treated with BA improved at a subsequent clinic visit. In some studies, higher clinical improvement rates (64–81%) have been noted in cases of *C. glabrata* vaginitis treated with BA [32,33]. In addition, one study reported successful therapy with topical 5-FU in 90% of women with *C. glabrata* vaginitis who had failed azole and BA therapies [33]. Moreover, it has been reported that the *in vitro* activity of 5-FU was superior to all other agents tested against *C. glabrata* [22]. However, it has also been shown that 7.1% (4/56) of *C. albicans* strains were resistant to 5-FU (MIC > 16 μg/ml) among patients with no history of previous

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exposure to this drug [20]. In our study, no resistant strains were detected, while 4.8% (10/207) of isolates (five *C. albicans*, four *C. glabrata*, and one *C. guilliermondii*) exhibited intermediate susceptibility to 5-FU (MICs of 8 and 16 μg/ml; Table 2). Additionally, CAS was included in this study because its topical use is relevant in the treatment of RVVC.

Extracellular hydrolytic enzymes appear to play an important role in VVC because these enzymes facilitate adherence and tissue penetration, leading to the invasion of the host [34–37]. In this investigation, significantly different percentages of *Candida* isolates derived from patients with AVVC or RVVC produced proteinase (74–92%) and phospholipase (49–74%) compared with the controls (28–50%) ($P < 0.05$; Table 3). Consequently, the proteinase activity of microorganisms on host cell surfaces is an important virulence factor because this activity facilitates the establishment of the infection, particularly of opportunistic fungi [36,37]. Thus, our study confirmed that proteinase activity played a more prominent role than other virulence factors in causing candidiasis [38,39]. In this study, there was a significant difference in phospholipase activity between the VVC (both acute and recurrent forms; 52–74%) and control isolates (28%) ($P < 0.05$). In addition, phospholipase production was found in the majority of *C. albicans* isolates in both AVVC and RVVC groups ($P < 0.05$). Previous studies have suggested that enzymatic secretion from *C. albicans* mucosal isolates is a determinant not only for pathogenic potential, but also for commensal colonisation of the mucosal environment by these yeasts [40,41].

In our study, the isolates derived from the symptomatic (63%) and asymptomatic (59%) RVVC groups were not more adherent to VECs than isolates from the AVVC (58%) and control groups (44%) ($P > 0.05$; Table 3). However, the ability to adhere to VECs was higher for the *C. albicans* strains than the non-*C. albicans Candida* strains ($P < 0.001$). Notably, interactions between yeast cells and epithelial cells comprise the primary step of pathogenesis for VVC [42,43]. Hemolytic activity was detected in most of the *Candida* isolates tested, and no significant differences were found among the organisms in the study groups 1–4 ($P > 0.05$). The lack of a correlation between hemolytic activity and RVVC pathogenesis suggests that this is not a relevant factor for pathogenesis [41,44].

Finally, this study addressed antifungal susceptibility profiles and virulence factors of yeast isolated from pregnant women with VVC, in whom there are constraints on treatment options due to potential fetal toxicity. The great majority of vaginal *Candida* isolates exhibited susceptibility to the antifungal drugs tested. We conclude that at least 90% of *Candida* isolates are susceptible to topical imidazoles *in vitro*; however, these drugs also require clinical trials investigating their use as treatment for infected women during pregnancy. Our results also suggests that proteinase and phospholipase production may have an important role in the pathogenesis of *Candida* vaginitis.

Contributions of authors

A. Kalkancı: design, coordination, investigation, laboratory tests, and writing; A.B. Güzel: clinical data, clinical sampling, and fungal culture; I.I.J. Khalil: laboratory tests; M. Aydın: laboratory tests; M. Ilkit: identification and deposition of yeast fungi, design, coordination, and writing; S. Kustimur: design and investigation.

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