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Prevalence, susceptibility profile and proteinase production of yeasts causing vulvovaginitis in Turkish women

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In this study the prevalence of vulvovaginal candidiasis (VVC), antifungal susceptibility and proteinase production of isolated *Candida* species were investigated. Vaginal swabs were collected from symptomatic women with vulvovaginitis attending the Obstetrics and Gynecology Clinic of Kocaeli University, Turkey. The relation between risk factors, such as pregnancy, diabetes mellitus, antibiotic and corticosteroid use, history of sexually transmitted diseases and contraceptive methods, was recorded. *Candida* spp. were identified by conventional methods, then evaluated for proteinase secretion in a medium containing casein. Antifungal susceptibility was determined according to the NCCLS microdilution method. The prevalence of women with vulvovaginitis was 35.7% (170/6080) and 16% (28/170) of them were diagnosed as VVC. *Candida albicans* was the dominant species: 21 (75%), followed by 4 *C. glabrata* (14%), 2 *C. tropicalis* (7%), and one *C. krusei* (3.5%). All isolates were susceptible to fluconazole, itraconazole and amphotericin B, except one *C. krusei,* one *C. glabrata* and one *C. albicans* that were resistant to fluconazole. Proteinase production was determined in 19 (90.5%) *C. albicans* and in all *C. tropicalis* isolates. Proteinase activity was not associated with antifungal resistance. No association was found between risk factors and VVC.

Key words: Vulvovaginal candidiasis; proteinase; antifungal susceptibility; risk factors.

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Vulvovaginal candidiasis (VVC) is a common disease affecting a large proportion of otherwise healthy women. It can be expected that 75% of all women will experience at least one episode of VVC during their life time, and approximately between 5 and 10% of these will experience at least three episodes of acute vaginal candidiasis within a 12-month period (1). VVC is responsible for 30% of all vulvovaginitis cases (2). Signs and symptoms of candidal vaginitis include vaginal and vulvar pruritus, erythema and/or edema, vaginal itching, burning, soreness, and dyspareunia (1). Discharge may be absent or physiological with a characteristic thick, white, curd-like or cottage-cheese-like appearance (1). Numerous risk factors have been associated with VVC, such as diabetes, pregnancy, and use of broad-spectrum antimicrobial agents, corticosteroids, and oral contraceptives (2).

A number of studies have concluded that *Candida albicans* is the major etiological agent of VVC (3, 4). It is currently suggested that non-albicans *Candida* spp. are increasing (5, 6), though only a few studies have produced data to support this claim. In specialist clinics >10% – and occasionally >20% – of patients

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have been infected with non-*albicans Candida* organisms (5, 6). The dominant non-*albicans Candida* sp. reported is *C. glabrata.* One possible reason for the apparent increase in non-albicans *Candida* vulvovaginitis is the increased use of antifungal therapy, sometimes inappropriately and frequently as a short, incomplete course of therapy, eliminating more sensitive *C. albicans* and selecting more resistant non-albicans *Candida* spp. It is possible that with the inappropriate use of multiple courses of azole therapy, resistant *Candida* isolates may emerge within vaginal colonizing yeasts in a manner similar to oropharyngeal candidiasis in HIV-seropositive patients (7, 8).

In the pathogenesis of VVC, several virulence factors of *Candida* spp. have been claimed. These putative virulence factors include especially adherence to host tissue, dimorphic transition, secretion of phospholipases and proteinases. Secretory aspartyl proteinases (Sap) may also play a major role in the pathogenesis of candidal vaginitis. These enzymes are implicated in the persistence and colonization of the vaginal tract (9, 10). They facilitate tissue penetration, causing infection and degradation of immunoglobulin A (IgA), which is an important factor in vaginal immunity (9, 11).

The purpose of this study was to determine: i) the prevalence of VVC and species distribution, ii) the relation between proteinase production and susceptibility profile of isolated *Candida* spp., iii) the relation between potential risk factors (pregnancy, diabetes mellitus, antibiotic and corticosteroid use, contraceptive methods) and VVC of symptomatic Turkish women with vulvovaginitis.

MATERIALS AND METHODS

Hospital

This study was conducted at Kocaeli University Hospital, Kocaeli, Turkey. Kocaeli University Hospital is a tertiary hospital serving the city of Kocaeli and its one million inhabitants. A total of 6,080 patients visited the outpatient clinic of the Department of Obstetrics and Gynecology between May 2002 and December 2003.

Patients

One hundred and seventy patients (age range 25–35 years) with signs and symptoms of vulvovaginitis were enrolled in the study. Inclusion criteria: symptoms of vaginal discharge, itching, frequent urination and/or irritation, and on gynecological examination signs of vulvovaginal edema, erythema and/or vulvar excoriation or fissure formation indicating vulvovaginal inflammation. Exclusion criteria: using antifungal drugs in the last 2 months and being menopausal. Standardized forms were used to record demographic characteristics, such as pregnancy, diabetes mellitus, long-term antibiotic or corticosteroid use, history of sexually transmitted diseases, contraceptive methods, symptoms and physical signs, and laboratory results. The study was approved by the Ethical Committee of Kocaeli University Hospital (No: 32/2001).

Case definition of VVC

VVC was defined as both a clinical diagnosis of VVC in patients with vaginal discharge and vulvar pruritus and growth of Candida spp. in culture of vaginal smear samples.

Vaginal samples

The samples were obtained from the lateral vaginal wall with sterile plain cotton-tipped swabs. One of the swab samples was immediately smeared on two glass slides, fixed, and sent to the laboratory for Gram staining. The other swab was mixed with 0.2 ml of sterile physiological saline in a test tube and also transported to the laboratory within 24 h for processing. Upon arrival at the laboratory, wet vaginal smears were treated with a drop of 10% KOH, covered with a coverslip, and examined for hyphal elements at 400×. Gram-stained preparations were also investigated for blastoconidia, pseudohyphae or mycelia.

Isolation and identification of Candida *spp.*

The vaginal swab samples were streaked onto Sabouraud dextrose agar (SDA) (Oxoid, UK), including chloramphenicol (50 μg/ml) and gentamycin (50 μg/ml) for isolation of *Candida* spp. After inoculation of vaginal samples, all plates were incubated at 35°C for 7 days. *C. albicans* strains were identified on the basis of chlamydoconidia formation in cornmeal-Tween 80 agar, production of germ tubes in serum, and growth on SDA containing 500 μg/ml cyclohexamide. The identity of isolated non-albicans *Candida* spp. was confirmed by sugar assimilation tests (API-20 C AUX; Bio Merieux, Marcy I'Etoile, France) (12).

Detection of proteinase activity

Secretory acid proteinase activities of all *Candida* isolates were tested using agar medium containing casein. For this purpose, first each *Candida* isolate grown in SDA was inoculated into YEPD medium (1% (wt/vol) yeast extract, 2% peptone, 2% dextrose) and then incubated at 30°C for 4 h. During the incubation period, casein agar medium (1% KH_2PO_4,

0.5% $MgSO_4$ (Difco, Kansas City, USA), 2% glucose (Oxoid, Hampshire, UK)) was prepared. The pH of the medium was adjusted to 5.0, then it was filter sterilized and added to a solution of autoclaved agar (2%). Upon cooling to 50°C, filter-sterilized casein (Difco, Kansas City, USA) was added to a concentration of 1%. Agar medium was poured into plates and they were left to dry. Filter paper disks, 6 mm in diameter, were placed on casein agar plates. A maximum of four discs per plate were used. Then, 10 µl of standardized inoculum (0.5 McFarland) of cultures grown in YEPD was dispensed onto disks. All agar plates prepared as above were incubated at 30°C for 6 days. For standard control, *C. albicans* CBS 2730 strain (Centralbureau voor Schimmelcultures, Baarn, The Netherlands) producing Sap was used (10, 13, 14) .

Sap activity was determined by observation of increased opacity around the disc and then measuring the lytic zone formed around the same disc. The results were scored according to the criteria of Rüchel et al. (15): (−) when negative or (±) when there was no visible or very limited clarification of the agar under the disc, (1+) when the zone of agar clarification was around (1–2 mm), and (2+) when the zone was >2 mm in diameter around the margin of the disc.

Antifungal agents

The following agents were supplied as standard powders: Amphotericin B (AMB) (Sigma-Aldrich, MO, USA), fluconazole (FLU) (Pfizer, NY, USA), itraconazole (ITR) (Janssen-Cilag, Beerse, Belgium). Stock solutions were prepared at 10 times the strength of the final concentration and diluted with RPMI 1640 (Sigma) with L-glutamine, without bicarbonate, supplemented with 2% dextrose and buffered to a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma) to obtain twice the final concentration.

Antifungal susceptibility testing

Candida isolates were tested by the NCCLS reference broth microdilution method (National Committee for Clinical Laboratory Standards, document M27-A2) (16). Two reference strains (C. parapsilosis, ATCC 22019; *C. krusei*, ATCC 6258) were included in each run for quality control.

Endpoint MICs

Minumum inhibitory concentrations (MICs) for FLU and ITR were defined as the first well with a significant reduction (approximately 50%) in growth compared to the positive control. For AMB it was defined as the lowest concentration able to inhibit any visual growth. The susceptibility breakpoints of FLU, ITR and AMB were ≤8 (µg/ml), ≤0.125 (µg/ml) and ≤1 (µg/ml), respectively. Endpoints of resistance for FLU, ITR and AMB were ≥64 (µg/ml), ≥1 (µg/ml) and ≥2 (µg/ml), respectively (16).

Statistical analysis

Association of Candida culture results with potential risk factors determined as categorical variables was identified by frequency analysis, Fisher's exact test and – when needed – Pearson's X2 test. The significance level was determined as 0.05. Data were analyzed using the SPSS 10.0 program for Windows.

RESULTS

Candida species were isolated by culture from 28 (16%) of 170 symptomatic patients. Only five of these culture-positive samples were wet-mount negative (18%); one of the samples was Gram-stain positive. Three samples were wet-mount and Gram-stain positive although they were culture negative.

A single yeast species was recovered from each culture. *C. albicans* was the most prevalent species: 21 (75%) followed by 4 *C. glabrata* (14%), 2 *C. tropicalis* (7%), and 1 *C. krusei* (3.5%). Amongst *Candida* species isolated, proteinase production was determined in 19 (90.5%) *C. albicans* and all *C. tropicalis* isolates. Only two isolates of *C. albicans*, four isolates of *C. glabrata* and one isolate of *C. krusei* were proteinase negative (Table 1).

Susceptibility patterns of *Candida* strains to FLU, ITR and AMB were also determined. The results obtained from the quality control strains indicated that our test was in the "good quality" range according to NCCLS recommendations. The MIC at which 50% of isolates are inhibited (MIC_{50}), MIC_{90} and MIC ranges are given in Table 2. The results showed that only one *C. albicans* and one *C. glabrata* strain was found to be resistant to FLU (MIC ≥64 µg/ml). MIC ranges for ITR and AMB were narrow: <0.03–2 and <0.03–0.5, respectively. *C. krusei* strain was evaluated as FLU resistant, regardless of its MIC result. MIC values for *C. krusei* were as follows: 2 µg/ml for ITR and 0.0625 µg/ml for AMB.

Potential risk factors for VVC are shown in Table 3. No significant relation was determined between all data and occurrence of VVC ($p>0.05$). In 146 non-pregnant patients, the most common method of contraception was coitus interruptus (n=70). The number of patients using other methods was determined to be low (oral contraceptive (n=3), hormonal injection

TABLE 1. *Proteinase production of* Candida *species isolated from patient with VVC*

Species	Degree of proteinase production: Total isolates N (%)[a]	Strong (2+) N (%)	Moderate (1+) N (%)	Negative (−) N (%)
C. albicans	21 (75.0)	17 (81)	2 (9.5)	2 (9.5)
C. glabrata	4 (14)	–[b]	–	4 (100)
C. tropicalis	2 (7.0)	1 (50)	1 (50)	
C. krusei	1 (3.5)			1 (100)
Total	28 (100)	18 (64.3)	3 (10.7)	7 (26)

[a] Percentages were calculated within species.
[b] Proteinase activity could not be detected.

TABLE 2. In vitro *susceptibility profile of* Candida *spp. isolates against three antifungal drugs*

		MIC (μg/ml)								
		Fluconazole			Itraconazole			Amphotericin B		
Species	(n)	range	50	90	range	50	90	range	50	90
C. albicans	(21)	0.06→64	0.25	4	<0.03–2	<0.031		<0.03–0.25	<0.03	<0.03
C. glabrata	(4)	0.25→64	1	>64	<0.03–1	<0.031		<0.03–0.5	<0.03	0.5
C. tropicalis	(2)	0.06–0.125	–	–	<0.03	–		–	–	–

TABLE 3. *The relation between several risk factors and vaginal candidiasis among symptomatic women*

Risk factors	Women with risk factors	Vulvovaginal candidiasis		P value
	n	n	%	
Pregnancy	24	5	20.8	0.2
Diabetes mellitus	5	2	40.0	0.5
[a]Previous use of antibiotic	11	3	27.3	0.6
Previous use of corticosteroid	34	2	5.8	0.2

[a] Last month before the visit.

(n=1), tube ligation (n=11)). Only the relation between VVC and cases using condom and intrauterine device (IUD) is shown in Table 4. Similarly, condom or IUD use was not associated with VVC ($p>0.05$). History of sexually transmitted diseases was determined in four patients (two gonorrhea and two syphilis).

DISCUSSION

Vulvovaginal candidiasis is one of the most commonly encountered diseases in Turkish women attending Obstetrics and Gynecology Clinics. Yeast may be present in the vagina despite the absence of clinical symptoms. In these patients, yeast is usually found in small numbers and predominantly in the blastoconidial form. In comparison, the presence of pseudohyphae/mycelia often tends to be associated with tissue invasion and is usually identified in symptomatic vaginal candidiasis (17).

Candida albicans is the major etiological agent in VVC, being responsible for approximately 90% of episodes of *Candida* vaginitis (15, 17). Other *Candida* species have been associated

TABLE 4. *The relation of vaginal candidiasis to IUD, condom, or use of other methods*

Cases	Condom users (n=25)		Methods other than condom (n=131)		IUD users (n=35)		Methods other than IUD (n=121)	
	n	%	n	%	n	%	n	%
VVC (+)	6	24.0	16	12.2	3	8.6	19	15.7
VVC (−)	19	76.0	115	87.8	32	91.4	102	84.3

with symptoms, particularly in patients subjected to several regimens of antifungal therapy. Although rare, azole resistance may be related to treatment failure of *Candida* vaginitis due to non-*albicans* species, such as *C. glabrata* (3–17).

In the present study we found that 16% of symptomatic women had clinical VVC and positive culture for yeast. Episodes of infection were mostly related to *C. albicans* (75%), followed by *C. glabrata* (14%). *C. albicans* may be considered the most virulent yeast species in the vagina followed by *C. glabrata* (18). The rather low prevalence of *C. albicans* and the high incidence of the non-*albicans* species contrasted with previous findings (19–22). However, low prevalences of *C. albicans* (60.7%) and *C. glabrata* (20.6%) in a Jordanian population (22) and similarly *C. albicans* (68.3%) and *C. glabrata* (8.9%) in a Belgian population (20) were also reported. Previous studies in our country showed that the prevalence of VVC in symptomatic women was between 11.3% (23) and 23.9% (24). In most instances, this low ratio has been correlated with prolonged antifungal therapy, mainly azole drugs. In our study, meanwhile, with the exception of one patient who had recurrent vaginitis due to fluconazole-resistant *C. albicans*, none of the patients had been undergoing azole therapy.

All *Candida* isolates obtained during the study were tested for antifungal susceptibility. As expected, all isolates were susceptible to AMB. The majority of the isolates were susceptible to FLU or ITR therapy as well. A study carried out on 84 symptomatic and 121 asymptomatic immunocompetent women showed that 10 isolates (5%) had dose-dependent susceptibility or resistance to azoles; and 7 of these species were non-*albicans* (21). Similarly, 13% of vaginal yeast isolates from Slovakia were reported to be resistant to FLU (25). In our study, only three isolates (10%) (one *C. albicans,* one *C. glabrata,* and one *C. krusei*) were determined to be resistant to FLU. Poor clinical and mycological outcomes are expected in patients infected with *C. glabrata*, for which, predictably, fluconazole MICs are above the concentration of FLU achievable in vaginal tissue (6, 26). *C. krusei* is an uncommon cause of VVC, in which there is intrinsic resistance to FLU. Although FLU resistance determined in this study was low, it should be viewed with concern and continued surveillance of antifungal susceptibility tests is needed so that we can monitor changing trends in the microbiology of VVC.

The recovery of FLU-resistant *C. albicans* isolates from vulvovaginitis patients was shown to be an usual event (19, 21, 27), but they often included small numbers of isolates. In the present study, a FLU-resistant *C. albicans* was isolated from a patient with systemic lupus erythematous. She was 35 years old and had been receiving deltacortril, chloroquine and imuran therapy for 10 years. She had two or three episodes in a period of one year and was treated with FLU for each episode. Thus, corticosteroid therapy and an underlying immunocompromising condition may explain the isolation of resistant *C. albicans*. Fidel & Sobel presented a complex model showing that the immunopathogenesis of recurrent candidal vaginitis is not caused by impaired systemic cell-mediated immunity; they postulated a host defect in local vaginal mucosa immunity (28). Sobel et al. (29) investigated the FLU susceptibilities of vaginal isolates of 556 women with complicated *Candida* vaginitis and its correlation with clinical response. The patients infected with resistant *C. albicans* strains appeared to respond clinically, as did those patients infected with highly susceptible strains. However, reduced mycological eradication was observed in patients infected with less susceptible strains (29). It was concluded that the patients infected with FLU-resistant *C. albicans* strains could be expected to improve clinically and respond to fluconazole treatment but would be more likely to remain colonized. These individuals would therefore remain at risk for a subsequent recurrence of symptomatic disease when conditions associated with relapse develop (29).

Apart from providing data on the prevalence and susceptibility profile of *Candida* spp., our study also describes the proteinase activities of *Candida* species isolated from VVC cases and their relation to antifungal susceptibility patterns. Several studies dealing with proteinase secretion have shown a clear correlation between the ability of *C. albicans* strains to secrete Saps and to cause disease (10, 14, 30, 31). In spite of this, there are only a limited number of reports on the correlation between antifungal susceptibility and virulence factors (19, 32, 33). Most *C. albicans* isolates (90.5%) were found to be

highly to moderately proteolytic, which is consistent with other published reports (10, 19, 34, 35). As in the present study, previous studies have reported proteinase activity by *C. tropicalis* (36, 37), but not by the species *C. krusei* and *C. glabrata* (37, 38). These results suggest that Sap production by *C. albicans* may be the crucial virulence factor and the pathogenesis of candidal vaginitis may depend on this enzyme. However, in this study it was shown that nonproteinase producers (*C. glabrata* and *C. krusei*) plus two *C. albicans* strains were also involved in vaginitis, indicating that Sap production is not the only essential factor in the pathogenesis of candidal vaginitis. On the other hand, both fluconazole-resistant isolates produced strong (2+) proteinase activities. Meanwhile, 75% of isolates secreting enzyme were not resistant to antifungal agents. *C. krusei* was resistant but also negative for proteinase. Thus, there seems to be no correlation between Sap and antifungal susceptibility.

Pregnancy, diabetes mellitus, antibiotic or corticosteroid use, IUD or oral contraceptives have been identified as risk factors for VVC. Nevertheless, there are studies reporting that IUD or oral contraceptives are not risk factors (39, 40). In our study, no statistical association was found between those risk factors and VVC. In cases with VVC, the number of IUD, condom or other contraceptive method users was lower than in cases without VVC. This result might be due to other factors with an adverse affect or low numbers of cases. An interesting point is that nearly 50% of nonpregnant women were using coitus interruptus. Unfortunately, this is still a commonly preferred method for Turkish women, especially those living in urban or rural areas (such as Kocaeli city) with low economic status. Use of this method may be a reason for vulvovaginitis due to VVC and/or other sexually transmitted diseases. Therefore, the contraceptive methods used by women with vulvovaginitis should also be investigated and an appropriate method should be recommended.

In conclusion, the high frequency with which *C. albicans* was recovered in this study and its azole susceptibility support the continued use of azole agents for empirical therapy of uncomplicated candidal vulvovaginitis. Laboratory tests, including species identification and antifungal susceptibility testing, should be requested only for a minority of a patients with recurrent VVC or an immunocompromised condition that leads to treatment failure.

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