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Original Article

Investigation of virulence factors and fluconazole resistance in strains isolated from candidal vaginitis in physiological menopausal and hysterectomized women

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Abstract

Objective: This research aims to evaluate the role of virulence factors of *Candida* species in vulvovaginal candidiasis (VVC).

Methods: In this research, *Candida* isolates obtained from physiological menopausal (n=30) and hysterectomized (n=30) women who were prediagnosed with VVC were collected for two years and identified according to conventional methods and API ID 32C assimilation profiles. The study examined the secreted acid proteinase (SAP) and phospholipase activities, slime production and phenotypic switching properties of these strains. In addition, the associations between these factors and possible resistance against fluconazole were investigated.

Results: The most commonly observed phenotypic switching pattern of strains was the "dwarf smooth" (DO) phenotype. Six strains (20%) in the physiological menopausal group and 13 in the hysterectomized group (43.3%) formed DO colonies. SAP activities, slime production of *Candida* isolates and phenotypic switching did not show statistically significant differences ($p>0.05$). The relationship between the number and production levels of phospholipase-producing strains and the phenotypic switching frequency was significant in the hysterectomized group compared to the physiological menopause group ($p<0.05$). All five strains with DO-forming ratios of ≤90% expressed at least one of the other virulence factors, whereas half of the 14 strains forming DO colonies at high ratios (91-100%) produced none of the other virulence factors. With an increase in the DO phenotypic switching ratio, the fluconazole MIC values (≥4 µg/mL) were also increased ($p<0.001$).

Conclusion: The phenotypic switching ability of *Candida* sp. may be an important factor in the pathogenesis of VVC and may lead to unresponsiveness to treatment.

Keywords: Fluconazole, menopause, phenotypic switching, vulvovaginal candidiasis.

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INTRODUCTION

Vulvovaginal candidiasis (VVC) is one of the most common clinical manifestations among female genital infections. Among women, 2/3 experience VVC at least once, and approximately 50% experience it more than once in their lifetime. Due to the reduction in estrogen levels in the postmenopausal period, even though the frequency of VVC is reduced compared to the fertility period, it is still identified in 1/3 of this group of patients. Previous studies have also reported that VVC is more common in menopausal women undergoing hormone therapy (1-3). The high recurrence rates of VVC in hysterectomized patients are explained as causative isolates being more resistant to treatment compared to the strains obtained from the physiological menopausal group. Although there are published data demonstrating the association between hysterectomy and the development of VVC, there is no study evaluating the role of virulence factors of causative yeasts in pathogenesis (4). Phospholipases, secreted acid proteinases and some other hydrolytic enzymes play a major role in the pathogenesis of *Candida* species. Degradation of extracellular hemoglobin, collagen, albumin, casein, some immunoglobulins and phospholipids of the host cell by these enzymes causes resistance to phagocytic killing mechanisms and may lead to more invasive infections (5). *Candida* strains exhibit spontaneous phenotypic switching at a frequency of 10^{-4}. This frequency may increase to 10^{-3}-10^{-1} depending on the conditions and the virulence of the strain. This change can be monitored in colonies, and it is known that a strain with a smooth-circular phenotype may switch to irregular-mycelial or white-opaque phenotypes (5).

The purpose of this study is to examine acid proteinase, phospholipase, slime production and phenotypic switching features of *Candida* strains isolated from VVC in physiological menopausal and hysterectomized women. Furthermore, it aims to determine interactions of these factors with each other and to investigate the association of fluconazole sensitivity of isolates with virulence factors in such groups.

MATERIALS AND METHODS

Candida isolates were obtained from physiologically menopausal women (n=30) and hysterectomized women (n=30) admitted to İstanbul Lütfi Kırdar Kartal Training and Research Hospital, Gynecology and Obstetrics Department over a two-year period. These women presented with at least two complaints of itching, burning sensation, redness, and cottage cheese-like discharge in the vulvovaginal region and were prediagnosed with VVC. Fifty-one of 60 isolates were identified as *C. albicans*, four as *C. glabrata*, three as *C. tropicalis* and two as *C. parapsilosis* according to germ tube production, appearance on Tween 80 cornmeal medium and API ID 32C assimilation profiles.

Secreted acid proteinase (SAP) production: SAP production of the isolates was examined on solid medium containing bovine serum albumin at pH 5.0. The *Candida albicans* CBS 2730 strain was used as a positive control (5).

Phospholipase production: Solid medium containing egg yolk (pH 4.2) was used to determine the phospholipase activities of the strains. The *C. albicans* SC 5314 strain served as a positive control (5).

Slime production: To detect the slime production of the strains, Christensen's "modified tube adherence method" was utilized. The *S. epidermidis* ATCC 35984 strain served as a positive control (6).

Phenotypic switching: Modified Lee's medium was utilized to examine the phenotypic switching of isolates (7, 8).

Susceptibility testing for fluconazole: For this purpose, the macro broth dilution method was used referring to the CLSI M27-A3/S3 document. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as standard strains (9).

Statistical analysis

Statistical analysis was conducted using SPSS 25.0 for Windows with a p value of 0.05. Frequency analysis was used to describe strains. Chi-square, trend chi-square and Fisher tests were utilized to compare binary data with a 95% confidence level.

RESULTS

Table 1 shows the distribution of *Candida* isolates according to patient groups.

Table 1. Distribution of *C. albicans* and nonalbicans isolates according to physiological menopausal and hysterectomized groups

Patient group	Species		Total n (%)
	C. albicans n (%)	Nonalbicans n (%)	
Physiological menopausal	28 (93.3%)	2 (6.7%)	30 (100%)
Hysterectomized	23 (76.7%)	7 (23.3%)	30 (100%)
Total	51 (85%)	9 (15%)	60 (100%)

Nonalbicans *Candida* strains were higher in the hysterectomized group, but this difference was not significant (p>0.05). The SAP production ratio was 86.6% in the physiological menopausal group, whereas it was 76.6% in the hysterectomized group and this result was not significant (p>0.05). The phospholipase production rate of isolates was 30% in the physiological menopausal group and 50% in the hysterectomized group, resulting in a significant difference (p<0.05). Slime production was 13.3% in the physiological menopausal group and 20% in the hysterectomized group (p>0.05). Isolates with phenotypic switching were 36.7% in the physiological menopausal group and 50% in the hysterectomized group (p>0.05). In the physiological menopausal group, 35.7% of the 28 *C. albicans* strains expressed phenotypic switching, whereas phenotypic switching was detected only in one of two nonalbicans strains, but this difference was not significant (p>0.05). In the hysterectomized group, phenotypic switching was observed in 34.8% of 23 *C. albicans* strains, while it was detected in all seven nonalbicans strains and this difference was significant (p<0.05). Table 2 shows the relationships between phenotypic switching ability and other virulence factors of isolates.

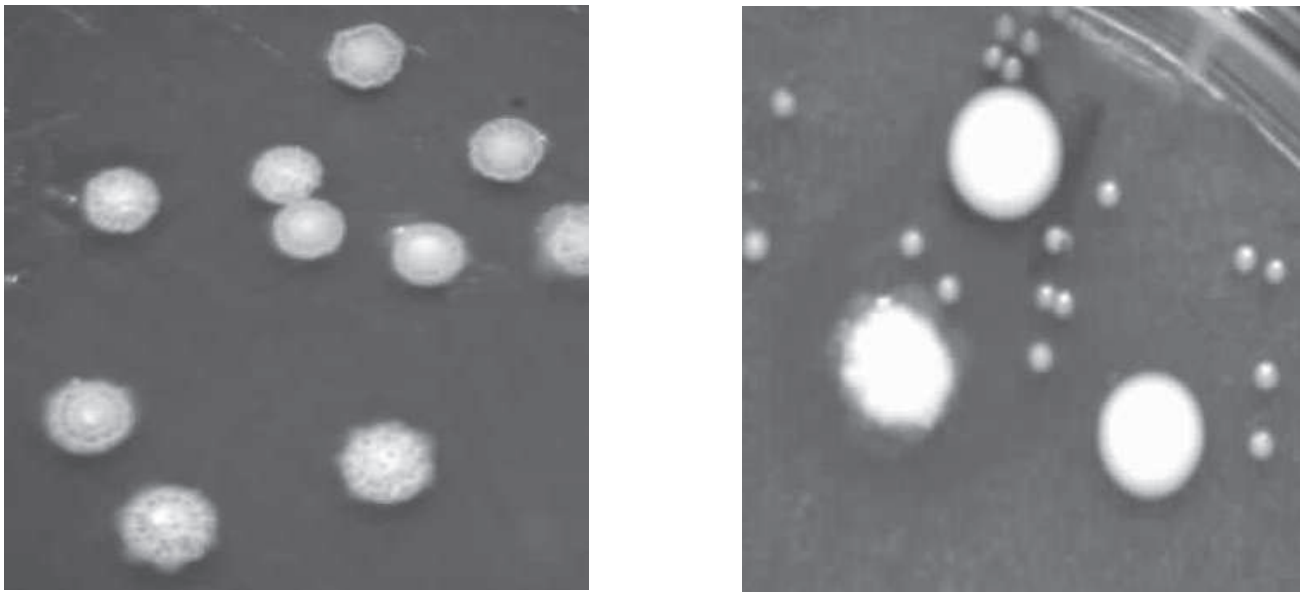
Table 2. The relationship between the presence of phenotypic switching and SAP, phospholipase, and slime production in isolates

Phenotypic switching	SAP[a]	Phospholipase[b]	Slime[c]	None[d]
Positive (n=26)	15 (57.7%)	6 (23%)	6 (23%)	7 (19%)
Negative (n=34)	34 (100%)	18 (53%)	4 (12%)	0 (0%)

Abbreviations: *[a]: SAP activity was detected in only half of the isolates that expressed phenotypic switching and in all that did not express, [b]: Phospholipase activity was detected in 23% of the strains that expressed phenotypic switching and in 23% that did not express, [c]: Slime production was detected in 23% of the strains that expressed phenotypic switching and in 12% that did not express, [d]: None of the other virulence factors were detected in seven out of 26 strains expressing phenotypic switching whereas all 34 strains not expressing phenotypic switching produced at least one of the other virulence factors.*

There was a significant positive relationship between SAP production and phenotypic switching (p<0.05). In contrast, there was a significant negative relationship between phospholipase production and phenotypic switching (p<0.05). In *C. albicans* and nonalbicans isolates forming smooth colonies under normal conditions, 6 different phenotypic switching patterns [dwarf-smooth (DO), mycelial (M), fuzzy (F), wrinkled (W), ring-wrinkled (RW) and wrinkled-mycelial (WM)] were detected (Figure 1). Of the 60 isolates, 26 (43%) underwent phenotypic switching.

Ten of these 26 strains formed colonies only in the DO phenotype. The remaining 16 strains exhibited DO and/or other phenotypes. Based on these results, DO is the most common switching phenotype. The patient groups were evaluated in terms of dominant phenotype DO colony forming rates. Only six strains (20%) formed DO colonies in the physiological menopausal group, whereas 13 strains (43.3%) formed DO colonies in the hysterectomized group. This difference was not significant (p>0.05). The isolates that exhibited phenotypic switching were divided into two groups based on DO colony forming rates. These groups were examined according to the presence of other virulence factors. All five isolates forming DO colonies at a rate of ≤90% exhibited at least one of the other virulence factors. In half of the DO colony-forming isolates that accounted for 91-100% (7/14), at least one of the other virulence factors was observed, while in the other half (7/14), none was detected. Due to the limited sample size, there was no significant difference between these two groups (p>0.05).



(A) **(B)**

Figure 1. Phenotypic switching patterns of the isolates on modified Lee's medium. A) RW and W colonies, B) O, DO and WM colonies (strain 60: *C. tropicalis* and strain 18: *C. albicans*, in the research of Türkölmez, N., Çerikçioğlu, N., 2010).

Table 3 shows the comparison of DO colony forming rates and MIC values of fluconazole.

Table 3. Comparison of DO colony forming ratios of the strains of physiological menopausal and hysterectomized groups and MIC values of fluconazole

DO colony forming rates	MIC Values			Total
	≥2 µg/ml	8 µg/ml	16 µg/ml	
100%	2 (20%)	2 (20%)	6 (60%)	10 (100%)
99-91%	2 (50%)	2 (50%)	-	4 (100%)
≤90%	43 (93.7%)	3 (6.5%)	-	46 (100%)
Total	47 (78.3%)	7 (11.7%)	6 (10%)	60 (100%)

Abbreviations: *DO: "dwarf smooth", MIC: Minimum inhibitory concentration*

DISCUSSION

Studies on VVC and recurrent vulvovaginal candidiasis (RVVC) in women of childbearing age have addressed the phenotypic switching properties of the causative fungi (10, 11). Although estrogen levels are reduced in postmenopausal women and secretory IgA continues to protect against infections in physiological menopausal women, VVC is observed in 1/3 of this group of women (2, 12). Research has reported that *C. albicans* ranks first in causative organisms in patient groups developing candidal vaginitis and is followed by other species (13-15). In our study, *C. albicans* was the most dominant pathogen in terms of the distribution of causative organisms. Although nonalbicans species were not common, they were all isolated from the hysterectomized group. Murta et al. conducted a retrospective study on hysterectomized women aged over 60 years who received hormone therapy. Their study showed that vaginal pH was more acidic and yeasts were detected at higher levels in smear tests compared to the physiological menopausal group. Authors have argued that this finding might explain the increasing incidence of VVC in hysterectomized women (16). There has been suggestion that *Candida* strains isolated from hysterectomized women are more resistant to treatment than those isolated from physiological menopausal women. Thus, it might be another reason that explains the high frequency of recurrent VVC in this group (4). There are studies examining the role of hydrolytic enzymes of causative fungi in women with VVC of childbearing age. Shinobu et al. reported that all *C. albicans* isolates (n=19) obtained from VVC cases were positive in terms of SAP enzymes (17). Consolaro et al. isolated *C. albicans* strains from patients with three different clinical conditions: asymptomatic carriers, vulvovaginal candidiasis (VVC) and recurrent vulvovaginal candidiasis (RVVC). Treatment with pepstatin A**,** which is an aspartate proteinase inhibitor**,** resulted in a reduction in the adherence of *C. albicans* to vaginal mucosal epithelial cells (53.1%, 48.7% and 59.9%**,** respectively). These results showed that SAP production might be an important virulence factor in the development of vaginitis (18). Lian and Liu investigated SAP 1-10 genes encoding secreted acid proteinases of *C. albicans* and their transcripts in the clinical materials of asymptomatic carriers, VVC and RVVC patients. SAP 2, SAP 4-6 and SAP 7 were determined to be the most predominant genes expressed in all three groups. The presence of SAP 2 and SAP 4-6 mRNAs was identified in all samples and SAP 7 mRNA was identified in some of them. SAP 1 and SAP 3 gene transcripts were found only in VVC and RVVC patients. In this study, researchers set forth that products encoded by different SAP genes were effective in cases of active disease and carrier state and anatomical localization of the isolates (19). In our study, 49 of 60 strains were SAP positive; however, there was no significant difference between the physiological menopausal and hysterectomized groups ($p>0.05$). Our findings are consistent with the data of these three studies and support the role of SAP in the pathogenesis of VVC. Regarding phospholipase, Shinobu et al. detected this enzyme activity in all 19 *C. albicans* strains causing VVC (17), an extensive study conducted in 2014 investigated various virulence factors of 46 strains isolated as the cause of VVC from women of childbearing age. Phospholipase activity is higher in VVC isolates than in blood isolates (20). In our study, the phospholipase enzyme was detected in a higher percentage of isolates in the hysterectomized group than in the physiological menopausal group and the difference was significant ($p<0.05$). The findings of both studies suggest that phospholipase might be a possible virulence factor that might explain the high incidence of RVVC in hysterectomized women. Chassot et al. observed that isolates from VVC patients tightly adhered to intrauterine devices (IUDs) *in vitro* and produced biofilms. Based on this observation, researchers suggested that the use of IUDs might be a factor in the development of VVC by supporting slime production (21). Kumar et al. reported that biofilm production, which they detected in 47.83% of 23 *C. albicans* isolates and in 81.25% of both 32 *C. parapsilosis* and 16 *C. glabrata* isolates obtained from women with VVC, might be important in the pathogenesis of VVC caused by nonalbicans strains (22). Slime production was detected in only 16.7% of our isolates and no significant difference was found between the two patient groups ($p>0.05$). There are no publications that can be compared with the postmenopausal period. Previous studies suggest that host factors and the phenotypic switching properties of causative yeast strains are important factors in RVVC cases. However, the studies were conducted in women of childbearing age and we could not find research concerning the importance of this factor in physiological menopausal and hysterectomized patient groups. Initial studies setting forth that phenotypic switching may be related to VVC were conducted in 1987 and 1989 by Soll et al. (10, 11). The researchers detected a high frequency (10^{-2}-10^{-3}) of phenotypic switching, mainly intensive mycelial and white-opaque phenotypes in nine of 11 *C. albicans* strains isolated from patients with recurrent vaginitis. They confirmed that primary isolates of the patients and derivatives that exhibited multiple switching profiles are the same strains with southern hybridization. They further suggested that phenotypic switching could be generated in the infection site as well (10, 11).

The present study found a higher phenotypic switching rate in the hysterectomized group isolates. Although this difference was not significant ($p>0.05$), the significance of phenotypic switching cannot be excluded in the hysterectomized group. Another notable finding is that while all nonalbicans strains in the hysterectomized group underwent phenotypic switching, this ratio remained at 34.8% for *C. albicans* strains. The difference was significant ($p<0.05$). Based on this finding, we can conclude that nonalbicans isolates are more prone to phenotypic switching in the hysterectomized group.

All 34 strains not exhibiting phenotypic switching produced at least one of the other virulence factors, whereas seven out of 26 strains that underwent phenotypic switching showed none of the virulence factors. SAP and phospholipase production in strains expressing phenotypic switching was significantly low ($p<0.05$). In the case of phenotypic switching, the production of both hydrolytic enzymes is reduced by at least half. This suggests a high possibility that yeast cells are efficient in metabolism for the production of virulence factors (Table 2). Among the seven different phenotypic switching types specified in the study, the most common type was DO, and it was at the limit of statistical significance in the hysterectomized group ($p=0.05$). Based on the results, this phenotypic switching makes the fungi comply with the changing physiological conditions of the host (23, 24). Regarding switching to the DO phenotype alone, all five strains with a DO-forming ratio of <90% expressed at least one of the other virulence factors, whereas only half of 14 strains that formed DO colonies in a high ratio (91-100%) produced any other virulence factors. This finding suggests that DO can be an important virulence factor on its own, despite lacking statistical significance, and the ones that do not have this ability may produce other virulence factors to comply with the microenvironment. Because the DO phenotype was the most common type of switching, the susceptibility profile of the strains against fluconazole within the frame of this phenotype was examined. As the switching to DO-type colony increased, MIC values of strains against fluconazole elevated as well. These results were significant with the trend chi-square test, which was used due to the low sampling size ($p<0.001$). These data suggest that the DO phenotype may be associated with microbiological and/or clinical unresponsiveness and recurrences that may develop (Table 3). Two studies conducted in 2000 and 2004 reported that susceptibility to fluconazole was reduced and resistance emerged in *C. glabrata* and *C. albicans* strains that were isolated from different areas of the body and exhibited dwarf colony mutation. Researchers reported that dwarf mutants developed respiration defects and had increased expression of CDR1 and CDR2 efflux pumps (25, 26). In this study, most *C. albicans* and nonalbicans strains that expressed the DO phenotypic switching phenomenon exhibited high MIC values against fluconazole, which can be explained by the same mechanism correlated with these studies. In a recent literature review, Nsenga and Bongomin have reported that recurrence in VVC is an increasing global health problem. Alternative therapies include topical amphotericin B and boric acid, although their use is constrained by their potentially harmful side effects. The oral echinocandin ibrexafungerp is effective at treating *Candida* vulvovaginitis and is well tolerated (27). In another study, Nasrollahi et al. reported that 49 (94%) of 52 isolates showed resistance to fluconazole. The *Pir1* gene was found to be overexpressed in 47 (96%) fluconazole-resistant *C. albicans* isolates (28). Dunaiski et al. reported that the high prevalence of *Candida* infections, particularly those caused by fluconazole-resistant nonalbicans *Candida* species, was quite concerning and could have negative effects on treatment (29). Yassin et al. stated that before choosing a treatment plan, an antifungal susceptibility test should be conducted due to the rise of resistant *Candida* strains (30).

Although the previous VVC and fluconazole treatment histories of the patients in the present study were not known, these findings regarding the isolates were considered to be associated with "possible" RVVC.

Limitations:

We could not increase the number of cases due to the very specific conditions, such as the limited number of hysterectomized and physiological menopaused cases with recurrent vulvovaginal candidiasis in a limited study time.

CONCLUSION

Based on this study data, particularly in the hysterectomized group, the followings are important factors in the etiology of RVVC: a higher number of *Candida* species other than albicans, the production of virulence factors such as phospholipase activity and phenotypic switching at high levels, mainly in DO-type colonies. However, DO-type switching colonies that were weakly positive for other virulence factors exhibited higher *in vitro* MIC

values against fluconazole. Therefore, it should be reminded that in the agents of RVVC, virulence is one of the mechanisms of recurrence rather than antifungal resistance, especially in hysterectomized women.

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