






Biofilm Formation and Aspartyl Proteinase Activity and Their Association with Azole Resistance Among *Candida albicans* Causing Vulvovaginal Candidiasis, Egypt

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Background: *Candida albicans* (*C. albicans*) is a major cause of vulvovaginal candidiasis (VVC), a condition that is commonly treated with azole agents. Biofilm formation and aspartyl proteinase production are important virulence factors that could be linked to azole resistance in *C. albicans* impeding therapy.

Aim: To find out the association of both factors with azole resistance among *C. albicans* isolated from VVC cases in Egyptian nonpregnant women of childbearing age.

Patients and Methods: In a cross-sectional study, *C. albicans* was isolated from nonpregnant females diagnosed clinically as having VVC during a 1-year study period. Susceptibility to azole agents was tested using the disc diffusion method. Biofilm formation and aspartyl proteinase production were assessed phenotypically. Additionally, two biofilm-related genes (*ALSI* and *HWPI*) and three proteinase genes (*SAP2*, *SAP4*, and *SAP6*) were screened for using polymerase chain reaction (PCR).

Results: Among 204 *C. albicans* isolates, azole resistance ratios were as follows: voriconazole (30.4%), itraconazole (17.6%), fluconazole (11.3%) and econazole (6.4%). Biofilm-producing capacity was detected in 63.2% of isolates, and 63.2% were proteinase producers. The frequencies of *ALSI* and *HWPI* were 69.6% and 74.5%, respectively, while *SAP2*, *SAP4*, and *SAP6* were 69.2%, 88.7%, and 64.7%, respectively. Biofilm formation was significantly associated with azole resistance ($P < 0.001$ for each tested azole agent) as was proteinase production ($P < 0.001$ for fluconazole, voriconazole, and econazole resistance and $P = 0.047$ for itraconazole).

Conclusion: Among nonpregnant Egyptian women of childbearing age, azole resistance in *C. albicans* causing VVC is significantly associated with biofilm formation and proteinase production. The development of new therapeutic agents that can target these factors is warranted.

Keywords: *Candida albicans*, vulvovaginal candidiasis, biofilm formation, aspartyl proteinase, azole resistance

Introduction

Vulvovaginal candidiasis (VCC) is a frequently encountered superficial fungal infection of the female lower genital tract characterized by itching, dyspareunia, soreness, and vaginal erythema and is caused by overgrowth of *Candida* species.¹

Being ranked as the second most common vaginal infection, after bacterial vaginosis, it was estimated that approximately 70–75% of women of childbearing age can experience at least one episode of VVC during their lifetime with a global yearly prevalence of 3871 per100000women.^{2,3}

Moreover, approximately 40% to 50% of VVC patients experience recurrence with 5% to 8% of them diagnosed with recurrent VVC "RVVC" (4 or more episodes per year),^{1,4} a condition associated with a high rate of therapeutic failure.⁵

The exact pathogenesis and risk factors of either VVC or RVVC are still to be elucidated as *Candida* species can colonize the lower genital tract of about 20% of healthy asymptomatic nonpregnant women forming part of vaginal microflora.⁶ However, and for reasons not fully understood, a transition to an infection state takes place. Factors that disrupt the vaginal microflora or interfere with the balanced host-*Candida* interplay can significantly increase the risk of VVC such as pregnancy, antibiotic usage, diabetes, and immunodeficiency.²

Candida albicans (*C. albicans*) continue to be the species most frequently (75–90%) recovered from cases of VVC.^{2,7–9} However, the frequency of non-*albicans* *Candida* (NAC), most notably *C. glabrata*, has been increasingly reported.¹⁰ Others include *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. guilliermondii*.^{11,12}

C. albicans possess a large repertoire of virulence factors that enable them to cause serious health problems such as their capacity to adhere to epithelial cells, biofilm production, secretion of hydrolytic enzymes, phenotypic switching, and hyphal formation.¹³

The growth of *C. albicans* in biofilm directly contributes to virulence as the complex architecture of the biofilm and the composition of the extracellular matrix in which cells are embedded enable the organism to withstand host immune defenses and high concentrations of antifungal drugs compared to planktonic cells.¹⁴ Biofilm formation is a sequential process that starts with adherence of yeast cells to the vaginal epithelium. This is followed by formation of several layers of polymorphic cells that are engaged in a self-produced extracellular matrix material composed mainly of β -1,3 glucan, and, finally, dispersion of unbound yeast cells to seed other sites.¹⁵ Upon this finely tuned process, the expression of nearly 1000 genes is upregulated including those coding for cell wall proteins that mediate adhesion to vaginal epithelial cells and to other *Candida* cells. Agglutinin-like sequence (*ALS*), a family of glycoproteins, constitutes the largest family of adhesins.¹⁶ The hyphal wall protein 1 (*HWPI*), a mannoprotein attached to the β glucan of the hyphal cell wall, constitutes another key protein that anchors *C. albicans* to epithelial cells. Both *ALS* and *HWPI* have a crucial role in mature biofilm formation, and their genes have been described to be upregulated during biofilm development.^{17,18}

Apart from biofilm formation, secreted hydrolytic enzymes by *C. albicans* such as aspartyl proteinases, first identified by Staib in 1969,¹⁹ have been regarded as important virulence factors as they facilitate host tissue invasion. Furthermore, they could be engaged in immune evasion, adhesion, hyphal development, and biofilm formation.²⁰ They include 10 members that are sequentially expressed at different stages of infection and are encoded by the aspartyl proteinases (*SAP*) gene family.²¹

The *SAP2* protein has been widely studied and has been shown to play a crucial role in vaginal infection.²² *SAP2* can degrade a wide spectrum of extracellular and host surface proteins such as keratin, collagen, and mucin.²¹ Interestingly and despite *SAP2*'s ability to degrade several host defense proteins such as immunoglobulins (Igs) and complement proteins, it can confer resistance against VVC and has been evaluated as a possible candidate for vaccine preparation.²³

Among the antifungals used to treat VVC, azole agents are the drugs of choice because of their utmost efficacy, availability in oral form, and lesser toxicity.²⁴ Azoles are five-membered heterocyclic compounds that exhibit their antifungal effect by binding to lanosterol 14- α -sterol demethylase, an important enzyme in ergosterol biosynthesis. This results in the synthesis of a fungistatic toxic sterol which, along with the ability of these agents to increase the level of reactive oxygen species, can inhibit fungal growth.²⁵

Unfortunately, a significant rise in the frequency of azole resistance among *C. albicans* strains has been reported and is regarded as a serious health issue that can impede VVC therapy.^{6,26} This is because it intensifies the therapeutic challenges caused by NAC species that naturally exhibit intrinsic decreased susceptibility to azole agents such as *C. krusei* and *C. glabrata*.²⁶ Furthermore, the development of resistance or tolerance to fluconazole in *C. albicans*, due to previous treatment, has been incriminated in the therapeutic failure of RVVC cases.⁵

This study aimed at studying the occurrence of biofilm formation and aspartyl proteinase production and their association with azole resistance in *C. albicans* causing VVC which is going to be of great utility in the proper understanding and management of VVC particularly resistant and/or RVVC cases.

Patients and Methods

This cross-sectional study was carried out over a period of one year (November 2021 to November 2022) at the Obstetrics and Gynecology Department, Zagazig University Hospitals, and Bacteriology and Molecular Biology

Laboratories at the Medical Microbiology and Immunology and Clinical Pathology Departments, Faculty of Medicine, Zagazig University.

Sample Size Determination

The sample size was determined using open EPI assuming the expected frequency of *Candida* infection was 31.6% among the target population (women with vaginitis).²⁷ At a 95% confidence interval and an effect size = 1, the estimated sample size was calculated to be 204 women.

Ethical Issues

The study protocol was approved by the Institutional Review Board of Zagazig University (approval no. IRB#: 9637). All patients completed informed consent, and all procedures involving human subjects were carried out in compliance with the updated 2013 Declaration of Helsinki.

Participants

Enrolled participants were recruited from patients attending the Gynecology Outpatient Clinics at Zagazig University Hospitals. Women (mean age 28.5 ± 6.13 years) suffering from symptoms of vulvovaginitis (burning, itching, curd-like discharge) were enrolled in this study. Pregnant women, those with cervical cancer, or were on antibiotics were excluded from the study.

Sampling and Identification of *C. albicans*

Two upper vaginal samples from each patient were collected using a sterile cotton swab. Within two hours, the samples were transferred and processed. Using one swab, a Gram-stained smear was prepared and examined to check for the presence of yeast cells, hyphae, and pseudohyphae and to exclude cases of bacterial vaginosis.²⁸ The other swab was inoculated onto a Sabouraud Dextrose Agar (SDA) plate (Oxoid Ltd., Hampshire, UK), with 50 µg/mL chloramphenicol, and incubated at 37°C for 24–48 h. Isolates of *C. albicans* were identified by assessing germ tube and chlamyospore formation.²⁹ This was confirmed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF/MS) using the VITEK MS system (bioMérieux, Inc, Durham, USA). Pure *C. albicans* growths were preserved in sterile glycerol broth (15% V/V) and kept at –20°C.

Antifungal Susceptibility Testing

According to the Clinical and Laboratory Standards Institute,³⁰ the susceptibility of *C. albicans* isolates to different antifungals was investigated by the disc diffusion method using cation-adjusted Mueller–Hinton agar (Oxoid, UK). The antifungal discs (Liofilchem, Italy) tested were amphotericin B (AMB, 100µg), fluconazole (FLU, 100 µg), voriconazole (VO, 1µg), itraconazole (ITC, 50 µg), caspofungin (CAS, 5µg), nystatin (NY, 100 µg), and econazole (ECN, 10 µg).

Phenotypic Detection of Biofilm Formation

This was done for all *C. albicans* isolates as previously described.^{31,32} Isolates were grown overnight on SDA plates, and fresh colonies were inoculated in Sabouraud dextrose broth (Oxoid, UK) and incubated for 24 h at 37°C and then adjusted (~1:100 dilution) to 0.5 McFarland standard using a fresh medium.³² Two hundred µL of the diluted cultures were added to each well of a sterile, polystyrene, flat-bottom microtiter plate (Techno Plastic Products, Switzerland), which was then covered with their lids and incubated for 48 h at 37°C. Sabouraud dextrose broth without *C. albicans* was used to inoculate negative control wells. The contents of the wells were then gently discarded by tapping the plate, and the wells were rinsed four times with 200 µL of phosphate-buffered saline (pH 7.2) to remove any free-floating planktonic cells. The plate was air-dried and 110 µL of 0.4% crystal violet solution (Fluka AG, Buchs, Switzerland) was added to each well to uniformly stain adherent cells. The plate was covered and kept for 45 minutes at room temperature before being thoroughly and repeatedly rinsed with distilled water. Stained biofilm with crystal violet was then solubilized in 200 µL of 95% ethanol, of which 100 µL was transferred to a new plate for reading. The optical density (OD) was measured using a microplate reader (Awareness Technologies stat fax 2100, CA, United States) at 595 nm. The isolates were tested in triplicate three times. The

mean OD \pm standard deviation (SD) was calculated for each tested isolate and for the control wells. The cut-off value of the OD (ODc) was calculated for each plate separately by adding the mean OD value of negative controls (mean ODnc) to three standard deviations of the negative controls ($3 \times \text{SDnc}$) using the formula: $\text{ODc} = \text{meanODnc} + (3 \times \text{SDnc})$. A negative result (non-producer) was recorded if the mean OD of the tested isolate was equal to or less than the ODc. Positive results were categorized as either weak ($\text{ODc} < \text{OD} \leq 2 \times \text{ODc}$), moderate ($2 \times \text{ODc} < \text{OD} \leq 4 \times \text{ODc}$), or strong biofilm producers ($4 \times \text{ODc} < \text{OD}$).³²

Phenotypic Detection of Aspartyl Proteinase Secretion

This was done according to Cassone et al with minor changes.³³ Yeast cells were incubated overnight in YEPD medium (2% glucose, 1% yeast extract, and 2% Bacto peptone) and induced to secrete proteases by subculture on bovine serum albumin (BSA) containing medium. To prepare the medium, 1.17% yeast carbon base (Sigma-Aldrich, India), 0.01% yeast extract (NICE, Manimala Road, Edappally, Kerala, India), and 0.2% BSA (Sigma-Aldrich, USA) were first adjusted to a pH of 5.0 and sterilized by filtration before adding to a stock solution of autoclaved agar (2%). Ten μL suspension of each isolate at a concentration of 10^7 yeast cells/mL in YEPD medium was applied to the plate. A maximum of 6 isolates were tested for each 90-mm-diameter plate. The plates were incubated at 28°C for 7 days and examined daily for increased opacity around the growing fungi caused by precipitated albumin which subsequently cleared due to hydrolysis by acid proteinases. The results were evaluated as negative (–) for no clearance, mild activity (a lysis zone of 1–2 mm around the growth), and strong activity (a lysis zone of 3–5 mm around the growth). *C. albicans* ATCC 10231 was used as a positive control and the test was completed in triplicate.

Detection of Biofilm and Aspartyl Proteinase Genes

Polymerase chain reaction (PCR) was applied to screen *C. albicans* isolates for the biofilm-associated genes “ALS1 and HWPI” and the proteinase genes “SAP2, SAP4, and SAP6” using previously described primers.^{34–36} DNA was extracted using the DNA extraction Mini Kit (QIAGEN DNA Mini Kit). Each PCR reaction was conducted in a total volume of 25 μL . The thermal conditions were as follows: 95 °C for 5 minutes, followed by 40 amplification cycles of 95 °C for 15s, 60 °C for 30s, and 68 °C for 40s, then a final elongation at 68°C for 5 minutes. Two PCR reactions with the same cycling conditions were applied to each isolate, the first targeting *HWPI*, *ALS1*, and *SAP2* and the second targeting *SAP4* and *SAP6*. The amplified PCR products were visualized in 2% agarose gel stained with ethidium bromide (0.5g/mL) using UV transillumination. A 100 bp DNA ladder was used to detect the size (bp) of the amplified genes.

Statistical Analysis

Collected data were analyzed using SPSS version 22 software (SpssInc, Chicago, ILL Company). Categorical data were presented as numbers and percentages. The Chi-square test (χ^2) was used to analyze categorical variables. $P < 0.05$ was considered significant.

Results

In this study, 204 non-duplicate *C. albicans* were isolated from an equal number of nonpregnant females suffering from vulvovaginitis. The susceptibility of the obtained *C. albicans* to the used antifungal agents is presented in [Figure 1](#).

All isolates were susceptible to nystatin and amphotericin B, and 93.6% were susceptible to caspofungin. Among azole derivatives, the highest resistance ratio was recorded with voriconazole (30.4%), followed by itraconazole (17.6%), fluconazole (11.3%), then econazole (6.4%).

The in vitro ability of *C. albicans* isolates to produce biofilm is demonstrated in [Figure 2](#). The results demonstrate that 63.2% of all isolates were biofilm producers where 14.7% ($n = 30$) were strong producers, 43.6% ($n = 89$) were moderate producers, and 4.9% ($n = 10$) were mild producers. However, 36.8% ($n = 75$) were non-producers.

The biofilm-related genes *ALS1* and *HWPI* ([Figure 3a](#)) were detected in 69.6% and 74.5% of isolates with a significant association between both genes and moderate and strong biofilm production, respectively ($P < 0.001$ and 0.002, respectively) ([Supplementary Table 1](#)).

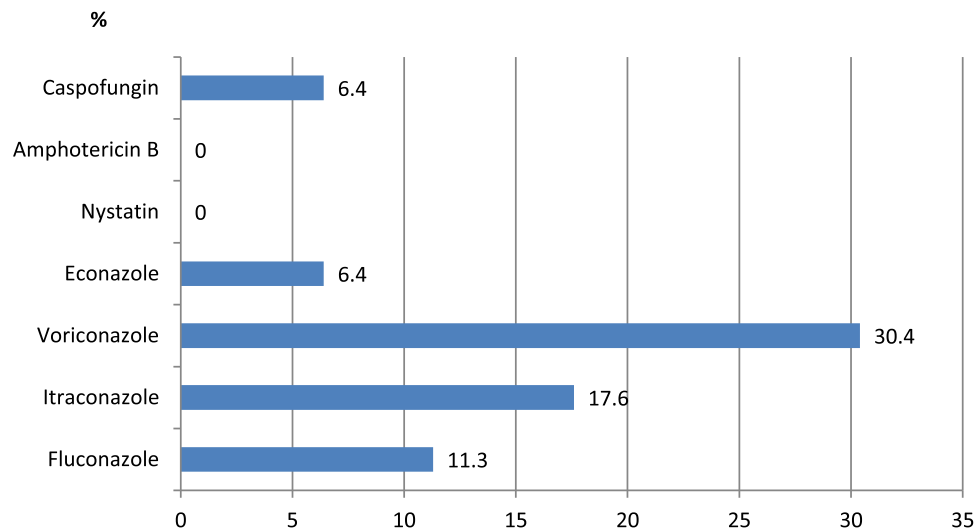


Figure 1 Frequency of antifungal resistance in the obtained *C. albicans* isolates (n = 204).

Notes: Resistance frequency of the tested antifungal agents among the obtained *C. albicans* isolates (n = 204). Regarding azoles, the highest resistance frequency was recorded with voriconazole (30.4%), followed by itraconazole (17.6%), fluconazole (11.3%), then econazole (6.4%). No resistance was recorded with nystatin or amphotericin B, and 6.4% were resistant to caspofungin.

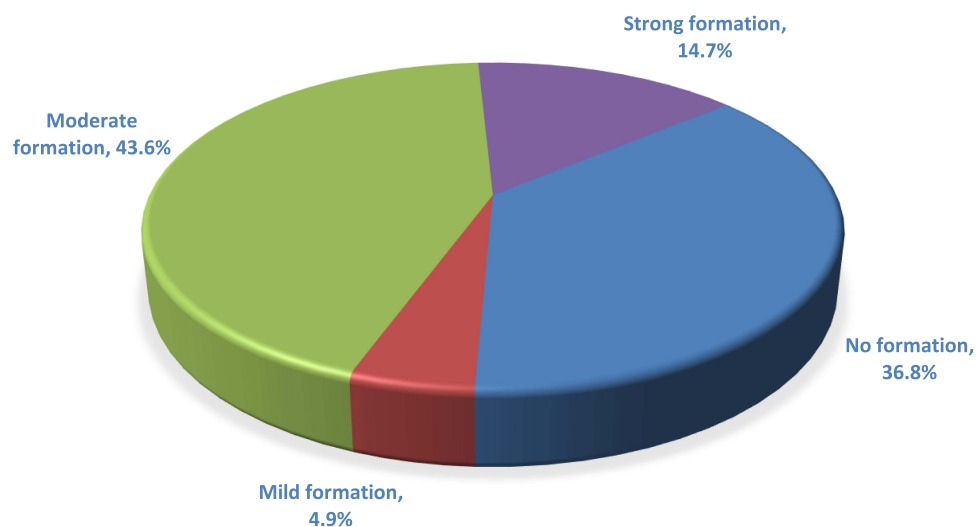


Figure 2 Biofilm formation among the obtained *C. albicans* isolates (n = 204).

Notes: A pie chart representing the frequency of biofilm formation among the obtained *C. albicans* (n = 204) as detected by the microtiter plate method. More than one-third of the isolates (36.8%) were non-producers. Biofilm producers (63.2%) were categorized into strong (14.7%), moderate (43.6%), and mild producers (4.9%).

When the relationship between biofilm formation and azole resistance was investigated, the results demonstrated highly significant relations with all tested azole drugs ($P < 0.001$ for each) (Table 1).

Among the obtained *C. albicans* isolates, 63.2% (n = 129) were able to produce aspartyl proteinases with 52.0% (n = 106) being mild producers and 11.3% (n = 23) being strong producers. The prevalence of three proteinase genes was tested and demonstrated that *SAP4* was the most frequent protease gene detected among all isolates (88.7%, n = 181), followed by *SAP2* (69.6%, n = 142), then *SAP6* (64.7%, n = 132) (Figure 3a and b). Furthermore, highly significant associations were found between the three virulence genes and the ability to produce aspartyl proteinases in vitro ($P < 0.001$ for each). Additionally, *SAP2* gene was found to be significantly associated with moderate and strong biofilm production ($P < 0.001$) (Supplementary Tables 2 and 3).

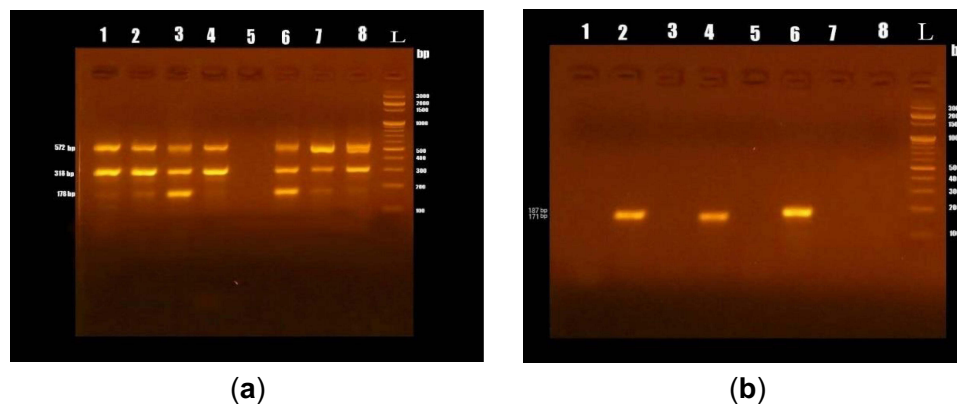


Figure 3 Gel electrophoresis of PCR targeting the biofilm-related genes *HWPI* (572 bp) and *ALS1* (318 bp) as well as the protease gene *SAP2* (178 bp) (a) and two other protease genes *SAP4* (171 bp) and *SAP6* (187 bp) (b).

Notes: (a) Lane L; 100 bp ladder; lane 5; negative control, lanes 1–4 and 6–8 demonstrate both the *HWPI* and *ALS1* genes, and lanes 3 and 6 demonstrate additionally the *SAP2* gene. (b) Lane L; 100 bp ladder, lane 8; negative control, lanes 2 and 4 demonstrate the *SAP4* gene, and lane 6 demonstrates the *SAP6* gene.

When the relationship between the production of proteinases and azole resistance was analyzed in the obtained isolates, highly significant associations with fluconazole, voriconazole, and econazole resistance ($P < 0.001$ for each) and a significant association with itraconazole resistance ($P = 0.047$) were found (Table 2).

Discussion

Despite being the drugs of choice for treating VVC, *Candida* species have evolved resistance to azole agents by different mechanisms, probably due to their fungistatic nature, resulting in therapeutic failures.³⁷ *C. albicans* possesses a plethora of virulence factors and several studies have found that azole resistance could be linked to these factors.^{38–40} In this study, the capacity of *C. albicans* to produce biofilms and aspartyl proteinases as well as the association of both factors with azole resistance was assessed in VVC isolates.

Among a total of 204 *C. albicans* isolates, 30.4%, 17.6%, 11.3%, and 6.4% were resistant to voriconazole, itraconazole, fluconazole, and econazole, respectively. Varying levels of azole resistance have been reported previously in *C. albicans*. This has been shown to depend on the type and site of infection, additionally, on the history of former or prolonged exposure

Table 1 Relationship Between Biofilm Formation and Azole Resistance in the Obtained *C. albicans* Isolates (n = 204)

		Biofilm Formation			χ^2	P
		None or Weak N = 85	Moderate N = 89	Strong N = 30		
Fluconazole	S	62 (72.9%)	89 (100%)	30 (100%)	36.3	<0.001**
	R	23 (27.1%)	0	0		
Itraconazole	S	49 (57.6%)	89 (100%)	30 (100%)	61.2	<0.001**
	R	36 (42.4%)	0	0		
Voriconazole	S	49 (72.9%)	63 (70.8%)	30 (100%)	18.9	<0.001**
	R	36 (42.4%)	26 (29.2%)	0		
Econazole	S	85 (100%)	76 (85.4%)	30 (100%)	17.9	<0.001**
	R	0	13 (14.6%)	0		

Note: **Highly significant.

Abbreviations: N, number; S, sensitive; R, resistant; χ^2 , Chi-square test.

Table 2 Relationship Between Aspartyl Proteinase Production and Azole Resistance in the Obtained *C. albicans* Isolates (n = 204)

		Proteinase Production			χ^2	P
		None N = 75	Moderate N = 106	Strong N = 23		
Fluconazole	S	75 (100%)	83 (78.3%)	23 (100%)	23.97	<0.001**
	R	0 (0.0%)	23 (21.7%)	0 (0.0%)		
Itraconazole	S	62 (82.7%)	83 (78.3%)	23 (100%)	6.12	0.047*
	R	13 (17.3%)	23 (21.7%)	0 (0.0%)		
Voriconazole	S	36 (48%)	83 (78.3%)	23 (100%)	30.4	<0.001**
	R	39 (52%)	23 (21.7%)	0 (0.0%)		
Econazole	S	62 (82.7%)	106 (100%)	23 (100%)	23.9	<0.001**
	R	13 (17.3%)	0 (0.0%)	0 (0.0%)		

Notes: *Significant; **Highly significant.

Abbreviations: N, number; S, sensitive; R, resistant; χ^2 , Chi-square test.

to these agents, particularly with fluconazole.⁴¹ Interestingly, it has been reported that invasive *Candida* members could be more susceptible to antifungals, including azoles, than those causing mucosal infection or colonization.^{42,43} In a previous Egyptian study, higher ratios of azole resistance were reported where 71% of *C. albicans* causing vulvovaginitis were resistant to voriconazole, 33% were resistant to itraconazole, and 27% were resistant to fluconazole.⁴⁴ In other studies, and consistent with the current study, the resistance ratios for fluconazole and voriconazole were 22.44% and 18.89%, in *Candida* species isolated from different clinical specimens and 14% and 34% in *C. albicans*.^{45,46} However, lower ratios were reported elsewhere.^{47,48}

In the current study, the susceptibility to antifungals other than azoles was tested due to their possible use as alternatives in resistant VVC cases.^{49–51} All isolates were susceptible to nystatin and amphotericin B and 93.6% were susceptible to caspofungin. This comes in accordance with previous studies.^{45,47,48}

C. albicans cells have been shown to grow mostly in biofilms, on both biotic and abiotic surfaces, which provide ecological advantages to the fungus and further constitute a source of recalcitrant infections.⁵² The current study demonstrated that 63.2% of *C. albicans* isolates were biofilm-producers with 14.7%, 43.6%, and 4.9% being strong, moderate, and mild producers, respectively. The prevalence of the biofilm-related genes *ALSI* and *HWPI* was 69.6% and 74.5%, respectively, with a significant relation between both genes and moderate and strong biofilm production, respectively (P < 0.001 and 0.002).

A previous study reported that among 53 *C. albicans* isolated from systemic infection, 22.6% were strong biofilm producers, 1.9% were moderate, 17% were weak producers, and 58.5% were non-biofilm producers.⁵³ The frequencies of *ALSI* and *HWPI* were 46.4% and 57.1% among 28 *C. albicans* causing catheter-associated candiduria in another study, where 32.1%, 21.4%, and 46.4% were strong, moderate, and weak biofilm producers, respectively. The authors documented that biofilm-forming isolates had a significantly higher prevalence of tested biofilm genes compared to the weak or non-biofilm-forming peers (P 0.049 and 0.001 for *ALSI* and *HWPI*, respectively),⁵⁴ which comes consistent with the current findings. Several factors could, however, influence in vitro biofilm production such as the isolation site of the tested strain, the initial inoculum size used in detection, and probably the substratum used whether silicone, polystyrene, polyvinyl chloride, or Teflon.^{55,56}

Though not fully understood, biofilm formation has been firmly connected to antifungal resistance. Different mechanisms have been suggested to explain this complex phenomenon with the composition of the biofilm matrix being probably the most important owing to its sponge-like effect that sequesters azole agents.⁵⁷ The present study recorded a highly significant association between biofilm formation and resistance to the tested azole agents (P < 0.001

for each agent). This comes consistent with previous reports that demonstrated a dramatic decrease in the susceptibility to antifungal agents of biofilm-associated *Candida*,⁵⁸ and that also reported significant negative correlations ($p < 0.05$) between fluconazole and voriconazole minimal inhibitory concentrations (MICs) and biofilm production in *C. albicans* isolates obtained from vulvovaginal specimens.³⁸ Similarly, significant relationships between fluconazole and itraconazole MICs and biofilm formation ($P = 0.05$ and $P = 0.002$, respectively) were reported in *C. albicans* isolated from other sites as nail samples.⁵⁹

However, such a significant correlation between antifungal drug resistance and biofilm formation was not reported by other researchers in *C. albicans* isolated from VVC and bloodstream infection.⁶⁰ Additionally, the in vivo ability to form biofilm was not directly connected to the level of fluconazole resistance in *C. albicans* clones isolated sequentially from an HIV-infected patient who received progressively increased fluconazole dosages over two years.⁶¹ This could be attributed to the different nature of the study and to the immune state of the studied patients which may interact with the organism in a complex way that affects the organism's virulence and pathogenesis.⁶²

Though remaining controversial,⁶³ the secretion of extracellular tissue-damaging hydrolytic enzymes such as proteinases seems to have a significant role in both invasive *Candida* infection and in mucosal infections such as vulvovaginitis. *C. albicans* isolates causing vaginitis were shown to produce a significantly higher level of aspartyl proteinases than isolates recovered from asymptomatic vaginal carriers.³⁴ In the current study, the ability to produce acid proteinases was demonstrated in 63.2% of *C. albicans* isolates with 11.3% being strong producers and 52.0% being mild producers. *SAP4* was the most frequent proteinase gene detected (88.7%), followed by *SAP2* (69.6%), then *SAP6* (64.7%) with highly significant associations between the three virulence genes and the ability to produce aspartyl proteinases in vitro ($P < 0.001$ for each).

High rates of proteinase production were reported previously in *C. albicans* whether in cutaneous or systemic infections. This ranged from 100% and 72.85% of *C. albicans* isolated from cutaneous infection and onychomycosis, respectively, to 90.74% with *C. albicans* causing surgical site infection.^{47,59,64} High rates were also reported in isolates obtained from those with impaired immunity such as HIV-infected individuals (92%) and diabetic patients with chronic periodontitis (94.5%).^{65,66} Regarding VVC, proteinase production has been shown to be one of the most expressed virulence determinants in *Candida* species (45%) following the adherence ability (100%).⁶⁷

The prevalence of proteinase genes in previous works demonstrated variable results. In *C. albicans* isolated from vulvovaginal colonization, *SAP6* was found to be the most frequent gene (93.33%), while *SAP7* was the most frequent (100%) in infection.⁶⁸ However, using quantitative real-time RT-PCR, another study reported that *SAP 2*, *SAP 4–6*, and *SAP 7* were the dominating proteinase genes expressed in both *Candida* carriers and patients with VVC and RVVC,⁶⁹ which comes in line with the current study. *SAP5* and *SAP9* were the most frequently expressed genes in vivo during mucosal infection (oral and vaginal), while the *SAP1* gene was the most prevalent in another study being found in 65% of *C. albicans* with a significant detection in biofilm-forming isolates ($P 0.0001$).^{48,70}

These obvious variations could be attributed to the differences in the type and site of infection as well as the stage of infection which may influence the expression of virulence factors both quantitatively and qualitatively.⁷¹ Additionally, variations in the in vitro culture conditions used for proteinase detection such as medium composition, pH of the medium, and temperature of incubation were found to have a notable effect on the induction and the activity of these enzymes.²¹

When the relation between the production of proteinases and azole resistance was analyzed in the current study, highly significant associations were found with fluconazole, voriconazole, and econazole resistance ($P < 0.001$ for each) and a significant association with itraconazole resistance ($P = 0.047$) was recorded.

Similar results were reported previously where in one study all isolates resistant to antifungals were aspartyl proteinase producers.⁵² However, in previous work on *C. albicans* isolates from onychomycosis, such a significant relationship was not reported with fluconazole ($P = 0.658$) or itraconazole ($P = 0.124$).⁵⁹ This could be attributed to the difference in the number of tested isolates, the different sites of infection, or probably the medium used for proteinase detection or the incubation conditions.^{72,73}

Limitations of the Study

This study is not without limitations such as the lack of assessment of the expression level of the studied genes to reveal their exact contribution to virulence and the lack of assessing both biofilm and proteinase as well as azole resistance in *C. albicans* colonizing normal females to compare those causing VVC and those inhabiting the vagina as microflora.

Data Sharing Statement

All data and materials related to the study are included in the current manuscript and in the [Supplementary Tables](#).

Ethical Approval and Consent to Participate

The study was approved by the Institutional Review Board (IRB), Faculty of Medicine, Zagazig University (Approval code 9637).

Disclosure

None of the authors have any conflicts of interest to declare for this work.

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