

# Extracellular Enzymes and Susceptibility to Fluconazole in *Candida* Strains Isolated From Patients With Vaginitis and Healthy Individuals

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**Background:** Extracellular phospholipase, proteinase, and coagulase are accounted as the major virulence factors in *Candida albicans*. Several reports showed that the incidence of resistance to fluconazole has risen during last two decades.

**Objectives:** The present study has investigated the extracellular enzymes of *C. albicans* and non-*albicans* species isolated from both patients with vaginitis and healthy women. In addition, susceptibility of the isolates was evaluated against fluconazole.

**Patients and Methods:** Vaginal samples were collected using sterile cotton swabs and inoculated on CHROMagar *Candida*. Routine morphological tests and ID 32C and API 20C AUX Kits were used to identify species. Phospholipase, proteinase, and coagulase activity were determined by standard methods. Susceptibility to fluconazole was also evaluated using ATB Fungus 3 Kits.

**Results:** The phospholipase activity was detected in 66.7% of the tested isolates recovered from patients with vaginitis. In the present study, phospholipase activity with higher Pz values (< 0.70) was more common in patients with vaginitis (28 of 66 isolates) whereas this rate in the normal individual was 13 of 42. Proteinase activity was detected in 74.2% and 61.9% of tested isolates recovered from patients and normal individuals, respectively. All tested isolates were negative for coagulase activity. In the present study, resistance to fluconazole was found in 34.8% of isolates. *C. dubliniensis* was the most common isolate (6 out of 11 isolates) that showed resistance to fluconazole.

**Conclusions:** Our results showed that *C. albicans* was the most frequently isolated from both patients with vaginitis and normal individual. In the present study, we could not find any correlation between extracellular activities and sources of isolates (patients and normal flora) and sensitivity or resistance to fluconazole.

**Keywords:** Vulvovaginal Candidiasis; *Candida albicans*; Phospholipase; Coagulase; Fluconazole

## 1. Background

*Candida* vaginitis is a common opportunistic infection that affects women during childbearing period, as well as patients with diabetes and immunodeficiency (1, 2). Vulvovaginal candidiasis is considered as a common opportunistic mycosis in female genital system. This disease usually affects 75% of women in their life time (3). It is usually associated with considerable morbidity, health-care cost, distress, pain, and sexual dysfunction (4). The predisposing factors such as immunodeficiency, diabetes mellitus, pregnancy, long-term treatment with corticosteroids, and oral contraceptives have an important role in the development of vulvovaginal candidiasis (2, 4).

*Candida albicans* is the most agent of *Candida* vaginitis followed by other species of *Candida* such as *C. glabrata* and *C. tropicalis* (4). Several reports have shown that the virulence of *C. albicans* is linked to pathogenicity factors such as adherence to epithelial cells, dimorphism (Y → M), cell surface composition, phenotypic switching, and thigmotropism (5-7). In addition, many virulence factors contribute to the pathogenicity of *Candida*, including production of extracellular enzymes (phospholipase,

proteinase, coagulase, esterase and hemolytic activity), biofilm formation, and surface adherence (8). Extracellular phospholipases and proteases have an important role in damaging fungal cell membranes. Both enzymes play an active role in the invasion of hyphal tip to enter the host cells.

Several topical and oral antifungals such as fluconazole, ketoconazole, clotrimazole, butoconazole, and miconazole are used for the treatment of *Candida* vaginitis (9). Resistance to antifungals is considered as an important factor for the recurrence and or delay in the treatment, especially in immunocompromised patients. Fluconazole is a triazole antifungal used for the treatment of *Candida* vaginitis as a single dose (9). Several reports show that the incidence of the resistance to fluconazole has risen during last two decades (9-12).

## 2. Objectives

The present study investigated extracellular phospholipase, proteinase, and coagulase activities in different isolates of *C. albicans* and non-*albicans* species isolated from both patients with vaginitis and healthy women. In

addition, susceptibility of the isolates was also evaluated against fluconazole.

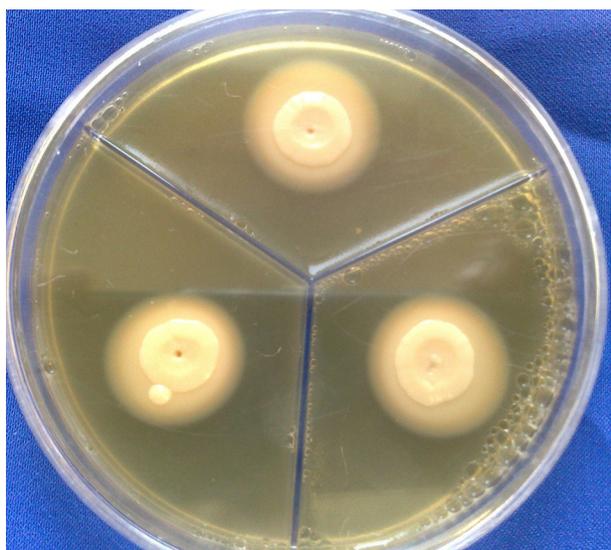
### 3. Patients and Methods

#### 3.1. Isolates and Preparation of Standard Suspension

Vaginal samples were collected using sterile cotton swabs and inoculated on CHROMagar Candida (CHROMagar Candida, France). Cultured media were aerobically incubated at 30 - 35°C for one week. Colonies producing a green coloration were presumptively identified as *C. albicans*. Furthermore, these species were confirmed by germ tube and chlamydoconidia formation. Other species were detected using CHROMagar™ Candida, ID 32C and API 20C AUX Kits (bioMérieux, France). A suspension of overnight cultures was prepared in phosphate-buffer solution (PBS) and adjusted with turbidity of 0.5 McFarland standards.

#### 3.2. Phospholipase Activity

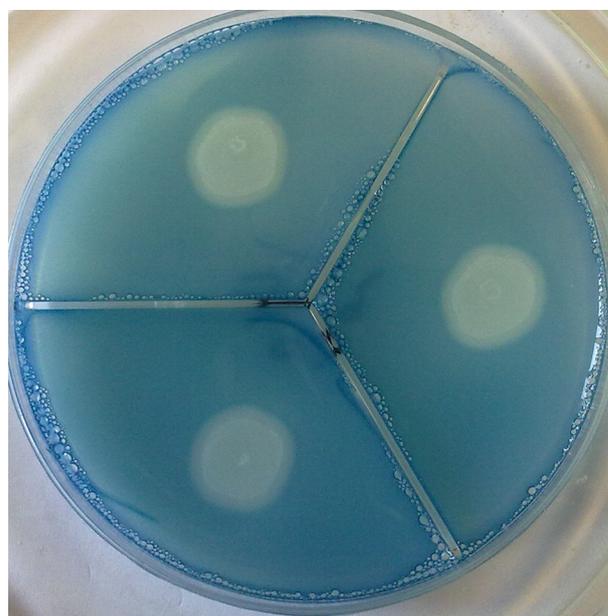
Phospholipase activity assays were performed according to Ge et al. (13) using Sabouraud dextrose agar (SDA, Merck, Germany) supplemented with egg yolk. Briefly, the test medium consisted of 6.5 g SDA, 5.84 g NaCl, 0.55 g CaCl<sub>2</sub>, and 2% egg yolk. Ten microliters of each standard suspension of strains was inoculated in triplicate and incubated at 25-27°C for 2 weeks (Figure 1). Colony diameter and the colony-plus precipitation zone were manually measured and the zone of phospholipase activity (Pz) was calculated. The Pz of 3 separate strains was measured and their average was calculated. The lower was the Pz value for each strain, the higher would be the phospholipase activity (14).



**Figure 1.** Phospholipase Precipitation Zone Around *C. albicans*

#### 3.3. Proteinase Activity

Proteinase activity of isolates was evaluated using bovine serum albumin agar medium (BSA) (15). Medium contained 1% agar (Merck, Germany), 0.1% (wt/vol) KH<sub>2</sub>PO<sub>4</sub>, 0.5% (wt/vol) MgSO<sub>4</sub>, 1% (wt/vol) glucose, and 0.16% (wt/vol) BSA as the sole nitrogen source, pH 5.0. Ten microliters of each standard suspension of tested strains was placed onto the test medium and incubated at room temperature for 2 weeks. All experiments were carried out in triplicate. Then, the plates were fixed with trichloroacetic acid (20%) and stained with 1.25% Amido black (CelloGel, Italy). The clear zone around each colony was manually measured and the Pz value for each 3 separate strains was calculated as described previously and finally the average Pz calculated (Figure 2).



**Figure 2.** Proteinase Clear Zone Around *C. albicans* (Stained With Amido Black)

#### 3.4. Coagulase Activity

The coagulase activity of the isolate was assessed using the rabbit plasma by a classical tube test. Zero point one milliliter of the culture of the isolate in Sabouraud glucose broth (Merck, Germany) was inoculated into a tube containing 0.5 mL of rabbit plasma and incubated at 30°C. A visible clot formation that could not be resuspended by gentle shaking at 2, 4, 6, and 24 hours was read as positive (5, 16). In addition, *Staphylococcus aureus* was used as positive control (5).

#### 3.5. Susceptibility to Fluconazole

Susceptibility to fluconazole was determined using ATB Fungus 3 Kits (bioMérieux, France) (17).

## 4. Results

In the present study, several species of *Candida* were isolated from patients with vulvovaginal candidiasis and healthy individuals. *C. albicans* (47.2%) was the most common species among the isolates followed by *C. glabrata* (24.1%), *C. dubliniensis* (12%) and *Candida* species (5.6%). Table 1 shows the details of the isolates recovered from patients with *Candida* vaginitis and healthy individuals.

**Table 1.** Frequency of Recovered *Candida* Species From Patients With *Candida* Vaginitis and Healthy Individuals <sup>a</sup>

<i>Candida</i> Spp	Healthy Individuals	Patients With <i>Candida</i> Vaginitis
<i>C. albicans</i>	19 (45.2)	32 (48.5)
<i>C. glabrata</i>	11 (26.2)	15 (22.7)
<i>C. dubliniensis</i>	2 (4.8)	11 (16.7)
<i>C. tropicalis</i>	1 (2.4)	1 (1.5)
<i>C. parapsilosis</i>	4 (9.5)	0 (0.0)
<i>C. guilliermondii</i>	0 (0.0)	1 (1.5)
<i>C. kefyfyr</i>	1 (2.4)	3 (4.5)
<i>C. humicola</i>	0 (0.0)	1 (1.5)
<i>Candida spp.</i>	4 (9.5)	2 (3.1)

<sup>a</sup> Data are presented as No. (%).

### 4.1. Phospholipase Activity

In the present study, all recovered yeasts were examined for the production of extracellular phospholipase. The phospholipase activity was detected in 66.7% and 54.8% tested isolates recovered from patients with vaginitis and normal individuals, respectively (Table 2). Phospholipase activity with higher Pz values (< 0.70) was more common in vaginitis (42.5%) whereas this rate in the normal individual was 31%. Thirty-three point three percent of tested isolates from patients with vaginitis were without phospholipase activity in comparison to 45.2% in normal flora isolates. In our study, all 9 strains of *C. parapsilosis* isolated from healthy individuals were without phospholipase activity.

### 4.2. Proteinase Activity

In the present study, out of 66 recovered isolates from patients with vaginitis, 49 (74.2%) isolates were able to show proteinase activity. Whereas 26 (61.9%) strains that were isolated from normal individual had proteinase activity (Table 3). About 45.5% and 28.6% of isolates were found to be positive for the production of extracellular proteinase with Pz, 0.80 - 0.89 (++) isolated from vaginitis and normal individual, respectively.

### 4.3. Coagulase Activity

In our study, all tested isolates from patients with vulvovaginal candidiasis and healthy individuals were negative for coagulase activity.

**Table 2.** Phospholipase Production in 108 Isolates of *Candida* Species From Patients With Vaginitis and Healthy Individuals <sup>a</sup>

<i>Candida</i> Spp	Healthy Individuals		Patients With Vaginitis	
	Negative	Positive	Negative	Positive
<i>C. albicans</i>	2 (4.8)	17 (40.5)	5 (6.1)	28 (42.4)
<i>C. dubliniensis</i>	0 (0.0)	2 (4.8)	1 (1.5)	10 (15.2)
<i>C. glabrata</i>	9 (21.4)	2 (4.8)	12 (18.2)	3 (4.5)
<i>C. tropicalis</i>	1 (2.4)	0 (0.0)	0 (0.0)	1 (1.5)
<i>C. parapsilosis</i>	9 (21.4)	0 (0.0)	0 (0.0)	0 (0.0)
<i>C. guilliermondii</i>	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)
<i>C. kefyfyr</i>	1 (2.4)	0 (0.0)	3 (4.5)	0 (0.0)
<i>C. humicola</i>	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)
<i>Candida spp</i>	2 (4.8)	2 (4.8)	1 (1.5)	1 (1.5)

<sup>a</sup> Data are presented as No. (%).

**Table 3.** Proteinase Production in 108 Isolates of *Candida* Species From Patients With Vaginitis and Healthy Individuals <sup>a</sup>

<i>Candida</i> Spp	Healthy Individuals		Patients With Vaginitis	
	Negative	Positive	Negative	Positive
<i>C. albicans</i>	7 (16.7)	12 (28.6)	8 (12.1)	24 (36.4)
<i>C. dubliniensis</i>	1 (2.4)	1 (2.4)	4 (6.1)	7 (10.6)
<i>C. glabrata</i>	7 (16.7)	4 (9.5)	3 (4.5)	12 (8.2)
<i>C. tropicalis</i>	0 (0.0)	1 (2.4)	1 (1.5)	0 (0.0)
<i>C. parapsilosis</i>	0 (0.0)	4 (9.5)	0 (0.0)	0 (0.0)
<i>C. guilliermondii</i>	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)
<i>C. kefyfyr</i>	0 (0.0)	1 (2.4)	0 (0.0)	3 (4.5)
<i>C. humicola</i>	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)
<i>Candida spp</i>	1 (2.4)	3 (7.1)	0 (0.0)	2 (3)

<sup>a</sup> Data are presented as No. (%).

### 4.4. Susceptibility to Fluconazole

We tested 66 isolates that were recovered from patients with *Candida* vulvovaginitis. In the present study, 34.8% of isolates were resistant to fluconazole whereas 53.1% and 12.1% were sensitive and dose dependent, respectively. *C. dubliniensis* was the most common isolate (6 of 11 isolates) that show resistance to fluconazole (Table 4).

**Table 4.** Sensitivity to Fluconazole Among 66 Isolates Recovered From Patients <sup>a</sup>

<i>Candida</i> Spp	Resistant	Dose Dependent	Sensitive
<i>C. albicans</i>	13 (19.7)	4 (6.1)	15 (22.7)
<i>C. dubliniensis</i>	6 (9.1)	1 (1.5)	4 (6.1)
<i>C. glabrata</i>	3 (4.5)	2 (3.1)	10 (15.2)
<i>C. tropicalis</i>	0 (0.0)	1 (1.5)	0 (0.0)
<i>C. guilliermondii</i>	0 (0.0)	0 (0.0)	1 (1.5)
<i>C. kefyfyr</i>	0 (0.0)	0 (0.0)	3 (4.5)
<i>C. humicola</i>	0 (0.0)	0 (0.0)	1 (1.5)
<i>Candida spp</i>	1 (1.5)	0 (0.0)	1 (1.5)

<sup>a</sup> Data are presented as No. (%).

## 5. Discussion

In a study conducted by Tay et al., the positivity rates for protease and phospholipase activities were 93.7% and 73.3%, respectively (18). Other studies showed that the levels of extracellular enzymes of phospholipase and protease activities in clinical isolates of *Candida* were found to be higher than those in commensal isolates (14,15). In a similar study, Ibrahim et al. found higher phospholipase activity in *C. albicans* isolates from the bloodstream in comparison to normal flora isolates of healthy individuals (19). Ombrella et al. have shown that 96.2% of vaginal isolates of *Candida* were positive for the production of phospholipase (15). On the other hand, in Vidotto et al. study, phospholipase and protease activity were found most frequently in oral cavity isolates (20). They believed that the productions of phospholipase and germ tube would accelerate the adhesion of *C. albicans* into membranes. Several reports have shown that the phospholipase activity of *C. albicans* was significantly higher than that of the non-*C. albicans* isolates (7, 8). However, Kantarcioglu and Yucel could not find any differences in phospholipase activities between *C. albicans* and non-*C. albicans* strains (21).

Proteinase activity of recovered isolates from respiratory (93%) and blood (83%) samples were significantly higher than that of urine (77%), pus (65%), and stool isolates (60%) (21). Our study shows that the secretion of both extracellular enzymes of protease and phospholipase in *Candida* species recovered from the patients with vaginitis was higher than healthy individuals. Rodrigues et al. have shown that the most of isolates of *C. albicans* (88.5%) and *C. tropicalis* (82.6%) from blood, stool, urine, respiratory and genital secretions were able to produce coagulase enzyme at 24 hours (5). In another study that conducted by Yigit et al., 64.7% of *C. albicans* strains induced clot formation (16). Whereas our study shows that all pathogenic and normal strains of *Candida* species were negative for the production of coagulase enzyme. There is little information concerning *Candida* coagulase activity in the literature. In addition coagulase activity in *Candida* species may be related to pathogenicity, which is poorly investigated and requires more rigorous studies (22). During recent years, an increase in non-*albicans* species, particularly *C. glabrata* and *C. tropicalis* as the etiology of vulvovaginal candidiasis were spotted (4, 23).

Several studies were shown that both species are more resistant to fluconazole compared to *C. albicans* strains resistance (10, 24). Fluconazole resistance was also observed among *C. glabrata* and *C. krusei* isolated from vaginitis in Richter et al. study (25). Sojakova et al. have believed that 13% of vaginal isolates of *Candida* were resistant to fluconazole (26). On the other hand, in a study conducted by Mohanty et al. 30 isolates of *Candida* (*C. glabrata*, *C. albicans*, *C. tropicalis* and *C. parapsilosis*) originated from vaginitis were evaluated against fluconazole. They found that none of the tested isolates were resistant to fluconazole (3). Our study shows that 34.8% of vaginal

isolates were resistant to fluconazole, including 13 of 32 *C. albicans*, and 10 of 34 non-*albicans* species. Some of the studies have shown that the level of extracellular enzymes in *C. albicans* isolates resistant to amphotericin B and fluconazole has increased (24, 27-29). However Kecli et al. have shown that there is no statistically correction between the phospholipase activity of 45 isolates of *C. albicans* and 5 isolates of non-*albicans* and resistant to fluconazole (29). Our study shows that there is not any significant correlation between the levels both extracellular enzymes and fluconazole sensitivity.

In conclusion, our results show that *C. albicans* was the most frequently isolated from both patients with vaginitis and normal individual. In the present study, we could not find any correlation between extracellular (phospholipase, proteinase, coagulase) activities and sources of isolates (patients and normal flora). However, the secretion of extracellular enzymes was higher in isolates from the patients compared to normal individuals.

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## Authors' Contributions

Ali Zarei Mahmoudabadi developed the original idea, the protocol, edited the final manuscript, and was guarantor. Zahra Seifi contributed to the development of the analyzed data, and prepared the manuscript draft. Majid Zarrin contributed to the development of the protocol.

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