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Original Article

Biofilm, proteinase and phospholipase production by *Candida albicans* isolates from a hospital in Thailand

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Abstract

This study aimed to determine biofilm formation, and production of phospholipase and proteinase in 73 clinical isolates of *Candida albicans*. Biofilms formed in a 96-well plate were quantified by crystal violet staining. Proteinase and phospholipase production levels were determined by spot assay on agar medium supplemented with the relevant substrates. In our study, all the isolates produced biofilm, and 89% were high biofilm producers. The mean biofilm amounts from each source of isolation ranged within 1.52 -3.41, and no significant difference of the mean values was detected. Over two-thirds (71%) of *C. albicans* isolates showed no proteinase production. By contrast, positive results for phospholipase activity were observed in 94% of the isolates. A highly significant correlation between biofilm formation and phospholipase production was detected. In conclusion, biofilm and phospholipase seem to be more prevalent compared to proteinase production. This suggests that phospholipase could potentially serve as a target for the treatment of *Candida* infection.

Keywords: biofilm formation, proteinase, phospholipase, *Candida albicans*

1. Introduction

Candidiasis is an infection caused by a yeast of the genus *Candida*. Most cases of candidiasis originate from overgrowth of *Candida* spp. living as normal flora in the gastrointestinal tract, vagina, urethra, and on the skin of patients (Spaulding *et al*., 2018; Tsui, Kong, & Jabra-Rizk, 2016). Non-*albicans Candida* species have shown a rising incidence of infections as a consequence of a reduced susceptibility to commonly used antifungal agents, but *Candida albicans* continues to be the most common causative agent causing candidiasis (Alkharashi *et al*., 2019). Virulence factors reported to be associated with *C. albicans* include: phenotypic switching, secreted hydrolytic enzyme production, adhesion, and capability of forming biofilm (Macias-Paz *et al*., 2023; Talapko *et al*., 2021). It is important to study virulence factors associated with infection in order to develop new treatments to overcome these yeast defense mechanisms. Biofilm formation, proteinase, and phospholipase are among the virulence factors most important in *C. albicans*. The structure of *Candida* biofilm containing yeast cells inside and covered with extracellular polysaccharide matrix can lead to resistance to antifungal drugs as well as host defense mechanisms (Eix & Nett, 2020). Since the major constituents of a host cell are proteins and phospholipids, proteinase and phospholipase are most likely involved in pathogenesis of *C. albicans* infection. Enzyme secretion facilitates invasion and colonization by causing rupture of the host cell membrane. Secreted proteinase of *C. albicans* is responsible not only for disrupting the host mucosal membrane but also for degrading immunological defense proteins (Monika, Małgorzata, & Zbigniew, 2017). The role of phospholipase involves hydrolyzing ester bonds in glycerophospholipids contributing to epithelial penetration of the host cell membrane (Nikou *et al*., 2019).

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Several studies have determined the proportions of *Candida* spp. expressing virulence factors (Guembe, Cruces, Peláez, Muñoz, & Bouza, 2017; Shirkhani, Sepahvand, Mirzaee, & Anbari, 2016). It has been found that the percentages of *C. albicans* isolates capable of forming biofilm and producing proteinase and phospholipase vary between studies. The fraction of *C. albicans* isolates capable of biofilm formation has been reported as 100 % (Sacristán *et al*., 2011), while in discordant results biofilm forming ability was as low as 8-12 % of the isolates in some other studies (Gokce, Cerikcioglu, & Yagci, 2007; Shin *et al*. 2002). Likewise, contradictory findings of *C. albicans* counts based on ability to produce hydrolytic enzymes have also been reported for both proteinase and phospholipase. Production of proteinase and phospholipase has been observed in most isolates of *C. albicans* (73 – 100%) in several studies (Bassyouni, Wegdan, Abdelmoneim, Said, & AboElnaga, 2015; Erum, Samad, Khan, & Kazmi, 2020; Lahkar, Saikia, Patgiri, Nath, & Das, 2017; Tay, Abidin, Hassan, & Ng, 2011). In contrast, other studies have found that only 29 – 57 % of the isolates were capable of enzyme production (Oksuz *et al*., 2007; Shirkhani, Sepahvand, Mirzaee, & Anbari, 2016).

The variations in those findings could be caused by biological differences of the tested isolates, type of infection, stage of infection, conditions used in experiments, as well as geographical origins. There are no previous studies from Thailand on the proportions *C. albicans* isolates with biofilm formation, phospholipase production and proteinase production. In this study, we therefore aimed to determine the abilities of *C. albicans* isolates from patients of Thammasat Hospital, Thailand, in biofilm formation, phospholipase production, and proteinase production. The patient body-sites of isolation were studied in order to broaden our understanding in the epidemiology of these isolates and to guide future efforts in control and prevention of infections due to *C. albicans*.

2. Materials and Methods

2.1 Microorganisms and growth conditions

Seventy-three clinical isolates of *C. albicans* obtained during October-November 2014 from hospitalized patients at Thammasat Hospital, Pathum Thani, Thailand, were used in this study. The isolates were identified by phenotypic methods including germ tube test and colony color on chromogenic media (HiCrome Candida Differential Agar, Himedia). Isolates with ambiguous results were further identified using ITS region sequencing according to manufacturer's service (Macrogen, Korea). The isolates were obtained from different fluids, depending on the site of infection: urine (n = 44), sputum (n = 14), blood (n = 6), pus (n = 6), and other locations (n = 3). The isolates were maintained in yeast extract-peptone dextrose medium (YPD medium: 1% yeast extract, 2% peptone, and 2% glucose) containing 20% glycerol at -80°C.

2.2 Biofilm formation assay

Biofilm was allowed to form on polystyrene 96-well plate using YNB medium containing 50 mM glucose as growth medium, as previously described (Pannanusorn, Fernandez, & Römling, 2013). Biofilm was quantified by crystal violet (CV) staining (Jin, Yip, Samaranayake, Yau, & Samaranayake, 2003). The amount of biofilm was determined spectrophotometrically by reading absorbance (A) at 595 nm of destained solution. The absorbance of each sample was subtracted from that of the blank (see below). The assay was carried out twice independently, and with three technical replicates each time. *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC22019 were included in the assay as positive and negative controls, respectively. A blank control well contained biofilm growth medium, but no yeast cells. Formation of biofilm was categorized to low biofilm (A_{595} <0.44), moderate biofilm (0.44 ≤ A_{595} ≤1.17), and high biofilm (A_{595} >1.17) according to the cut-offs for classifying the ability of biofilm formation in *Candida* spp. (Marcos-Zambrano, Escribano, Bouza, & Guinea, 2014).

2.3 Secreted proteinase assay

Assessment of proteinase production was performed using a spot assay on agar plate containing bovine serum albumin (BSA) as protein supplement, as previously described (Pannanusorn *et al*., 2014). The clear zone around a colony indicating the proteolytic activity was observed after staining with amido black dye. The ratio of diameter of colony (A) to that of clear zone (B) was determined as the precipitation zone (Pz). Degree of proteolytic activity was classified as no activity (Pz = 1), low activity (0.64 ≤ Pz <1), and high activity (Pz < 0.64) (Ozkan, Kaynak, Kalkanci, Abbasoglu, & Kustimur, 2005). Each isolate of *C. albicans* was tested in triplicate in two independent experiments. *C. albicans* ATCC10231 was used as a positive control, while a strain from our laboratory, *C. albicans* U506/10 (in this publication), was used as a negative control.

2.4 Phospholipase assay

The ability of yeast cells to produce phospholipase was assessed using a spot assay on SDA agar containing 1 M NaCl, 5 mM $CaCl_2$, and 8% egg yolk (Oxoid), as previously described (Borst & Fluit, 2003). The ratio of the diameter of colony (A) to that of opaque zone (B) was determined as the precipitation zone (Pz), used to express enzyme activity. Degree of phospholipase activity was classified as no activity (Pz = 1), low activity (0.64 ≤ Pz <1), and high activity (Pz < 0.64) (Ozkan *et al*., 2005). The assay was carried out in three replicates in two independent experiments. *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 were used as positive and negative controls, respectively.

2.5 Statistical analysis

The difference of biofilm formation of the categorized groups was tested using the unpaired *t*-test. Biofilm formation and hydrolytic enzyme production of *C. albicans* isolates were tested using one-way analysis of variance (ANOVA). The distribution of isolates according to ability of hydrolytic enzyme production was tested using the χ^2 test. Biofilm formation, proteinase and phospholipase production of the isolates were checked for association using correlation test. GraphPad Prism version 5 (GraphPad Software, San Diego, CA. USA) was used for statistical

analysis. A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Biofilm formation of *C. albicans* isolates

Biofilm was spectrophotometrically determined by CV staining. The isolates tested in this study showed highly variable biofilm forming ability. Under the conditions used in this study, 89% of isolates were high biofilm producers, while only 11% of the isolates were moderate biofilm producer. Low biofilm or no biofilm producers were not detected in our study. The differences in biofilm formation were statistically significant between the categorized isolates ($P < 0.0001$) (Table 1).

Biofilm formation was further characterized with regard to the site of infection. The isolates obtained from pus, blood, and other sources showed only high biofilm formation. Although the isolates from pus possessed the highest mean biofilm formation compared to biofilms formed by *C. albicans* isolates from other sites, no significant difference in the mean values was detected ($P > 0.05$) (Figure 1).

Table 1. Counts of *C. albicans* isolates according to biofilm formation ability

Sample type (number of isolates)	Number of isolates		
	High biofilm producer (%)	Moderate biofilm producer (%)	Low biofilm producer (%)
Urine (44)	37 (84)	7 (16)	-
Sputum (14)	13 (93)	1 (7)	-
Pus (6)	6 (100)	-	-
Blood (6)	6 (100)	-	-
Others (3)	3 (100)	-	-
Total (73)	65 (89)	8 (11)	0 (0)

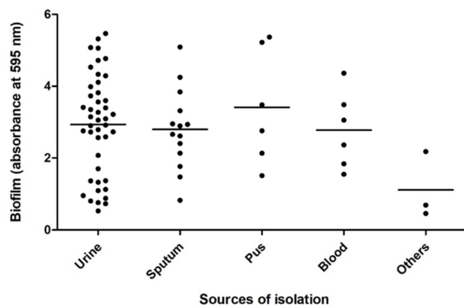

Figure 1. The 48- h biofilms formed on 96-well plates by 73 *C. albicans* isolates, grouped according to source of isolation

3.2 Proteinase and phospholipase assay

The capability of *C. albicans* isolates to produce hydrolytic enzymes were categorized as high, low, and no enzyme production according to calculated Pz value described above. The clear zone surrounding the colony after protein destaining indicates proteinase activity. The isolates of *C. albicans* were significantly less frequently observed for proteinase production (21/73, 29%) ($P < 0.001$). Conversely, phospholipase activity was more frequently observed among the tested *C. albicans* isolates (69/73, 94%) ($P < 0.001$). However, most isolates with positive phospholipase activity were grouped in the moderate production (41/69, 59%). In addition, we further characterized whether ability to produce hydrolytic enzymes is different in *C. albicans* isolated from different sites of infection. Our results revealed that neither proteinase nor phospholipase production was statistically different by site of *C. albicans* infection (Table 2 and Figure 2).

Table 2. Proteinase and phospholipase production among *C. albicans* isolates

Hydrolytic enzyme assay (*n*, % isolates positive for variable by type of source)	Number of isolates		
	High activity (%)	Low activity (%)	No activity (%)
Proteinase			
Urine (13, 29 %)	8 (18)	5 (11)	31 (71)
Sputum (3, 21 %)	2 (14)	1 (7)	11 (79)
Pus (2, 33 %)	0 (0)	2 (33)	4 (67)
Blood (1, 17 %)	0 (0)	1 (17)	5 (83)
Others (2, 67 %)	2 (67)	0 (0)	1 (33)
Total (21, 29 %)	12 (17)	9 (12)	52 (71)
Phospholipase			
Urine (42, 95 %)	19 (43)	23 (52)	2 (5)
Sputum (13, 93 %)	5 (36)	8 (57)	1 (7)
Pus (6, 100 %)	1 (17)	5 (83)	0 (0)
Blood (5, 83 %)	1 (17)	4 (66)	1 (17)
Others (3, 100 %)	2 (100)	1 (100)	0 (0)
Total (69, 94 %)	28 (38)	41 (56)	4 (6)

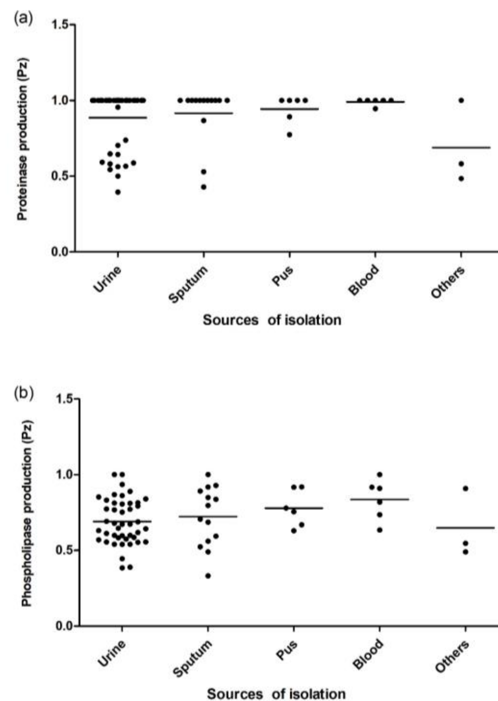

Figure 2. Proteinase production (a), and phospholipase production (b) of 73 isolates of *C. albicans* grouped according to source of isolation

3.3 Correlation of biofilm and hydrolytic enzymes

Correlation of biofilm forming ability, proteinase production and phospholipase production was analyzed for overall *C. albicans* isolates and in the groups of isolates according to the sites of infection (*C. albicans* isolates obtained from other sources were not included due to a small sample size (n=3)) (Table 3). No correlation of biofilm forming ability with proteinase production was detected in the 73 isolates of *C. albicans* overall. However, a weak correlation of biofilm formation and proteinase production was detected specifically in the isolates obtained from pus. In contrast, a highly significant correlation between biofilm formation and phospholipase production was detected in the 73 isolates of *C. albicans* overall, although no correlation was detected in the isolates from sputum specifically. Lastly, only the isolates from pus exhibited a significant correlation between proteinase and phospholipase production.

Table 3. Significances of correlations between biofilm formation, proteinase and phospholipase production in *C. albicans* isolates

C. albicans according to site of isolation	Significance of correlation		
	Biofilm - Proteinase	Biofilm - Phospholipase	Proteinase - Phospholipase
C. albicans (73)	ns	***	ns
Urine (44)	ns	**	ns
Sputum (14)	ns	ns	ns
Pus (6)	*	***	*
Blood (6)	ns	***	ns
Others (3)	ND	ND	ND

Significant difference with *, 0.01< P <0.05; **, 0.005< P <0.01 and ***, P < 0.005, ns, not significant, ND, not determined due to a small sample size

4. Discussion

Biofilm is not a stable characteristic of *Candida*. Several environmental factors *in vitro* can affect the formation of biofilm. Some of the most notable factors are type of medium (Leonhard, Zatorska, Moser, Tan, & Schneider-Stickler, 2018; Weerasekera *et al*., 2016), shear stress (Mukherjee, Chand, Chandra, Anderson, & Ghannoum, 2009), and surface conditions including surface hydrophobicity, roughness, and pre-conditioning (De-la-Pinta *et al*., 2019; Frade & Arthington-Skaggs, 2010). In our study, we used defined YNB medium with 50 mM glucose for biofilm development (Al-Fattani & Douglas, 2006). Our previous study has shown no differences in biofilm formation when using YNB medium with 50 mM glucose compared to using Sabouraud Dextrose Broth (SDB) with 8% glucose as a culture medium (Pannanusorn *et al*., 2013). RPMI 1640 is another commonly used medium for *Candida* biofilm experiments. However, RPMI 1640 medium induces hyphal formation (Kucharikova, Tournu, Lagrou, Van Dijck, & Bujdakova, 2011), so this medium definitely has an effect on biofilm mass. In order to obtain relevant and comparable information, we therefore used YNB medium with 50 mM glucose in our study. It should be noted, however, that this medium did not mimic the source of isolation and consequently may have had an effect on the amount of biofilm.

Capability of forming biofilm is among the important virulence factors of *C. albicans*. Under conditions used in our study, biofilm formation was detected in all isolates tested, and 89% of the isolates produced a high amount of biofilm. This finding differs from several studies, which have often reported significantly lower proportion of *C. albicans* isolates with biofilm forming ability compared to non-albicans *Candida* spp. Our result was, however, in agreement with a recent report which showed that all the tested isolates (n= 41) of *C. albicans* were capable of biofilm formation, and 92% (n= 38) were categorized as a moderate-to-high biofilm producers (Guembe *et al*., 2017).

Regarding the sites of infection, we also showed here that capability of forming biofilm in the isolates recovered from blood stream infection was comparable to ones recovered from other sites of infection. This is concordant to a previous report (Shin *et al*., 2002) and supports the suggestion that other attributes rather than biofilm contribute to *Candida* infection (Jin *et al*., 2003). Moreover, comparison of the average biofilm value from different sites of infection revealed no statistically significant differences in our study. The isolates from either sterile (blood samples) and non-sterile sites (urine, sputum, pus samples) showed similar values of average biofilm. This finding is, in part, consistent with a study that showed no differences in biofilm formation between oral and vaginal isolates of *C. albicans* (Li, Yan, & Xu, 2003). In contrast to our results, the study by Guembe and colleagues (Guembe *et al*., 2017) reported that high biofilm formation was more observed in the isolates of *Candida* species from sterile sites (sterile fluids, biopsy specimens and catheter samples) than in those from the non-sterile sites (urine and respiratory tract samples). Overall, it is not possible to conclude whether biofilm formation of *C. albicans* is associated with the source of isolation since there is insufficient information either from our study or the previous studies. More importantly, one notable fact is that biofilm formation is greatly variable between isolates of *Candida* spp. regardless of the site of infection. Our study, therefore, underlines this important characteristic of biofilm formation by *C. albicans*.

Phospholipid and protein are the main constitutes of human cell membrane, and definitely they are targets of enzyme attacks. Phospholipase of *C. albicans* is suggested to play an important role in an early step of host invasion by hydrolyzing one or more ester linkages in glycerophospholipids (Ghannoum, 2000). There are four different classes (A, B, C, and D) of phospholipase in *C. albicans* (Ghannoum, 2000; Mayer, 2013). However, only class B phospholipase (PLB) is extracellular, as when the enzyme activity is observed from a precipitation zone around the colony grown on agar plate containing egg yolk as substrate (Ghannoum, 2000). Phospholipase B gene (*PLB*) of *C. albicans* is a multigene family comprising five members of phospholipase (*PLB1-5*). The *PLB1, PLB2,* and *PLB5* have been characterized in detail. Interestingly, the study on *PLB5* suggested that *PLB5* contains a glycosylphosphatidylinositol (GPI) anchor attachment site and therefore encodes a cell-associated phospholipase. Comparing phospholipase activity between *C. albicans* wild type strain and isogenic *PLB5*

mutants (*plb5* Δ/Δ), however, did not show significant differences. Furthermore, whether *PLB5* is involved in virulence is poorly understood (Theiss *et al*., 2006). Several studies on *PBL1* and *PBL2* gene disruption have shown that most likely only *PLB1* is responsible for *C. albicans* pathogenicity (Mukherjee *et al*., 2001; Naglik *et al*. 2003)

Here, we reported a relatively high number of isolates (approximately 95%) with positive phospholipase. This is consistent with other reports in that most isolates of *C. albicans* can produce phospholipase (Erum, Samad, Khan, & Kazmi, 2020; Lahkar, Saikia, Patgiri, Nath, & Das, 2017;). Therefore, our results support the fact that phospholipase is a significant virulence factor in *C. albicans*. In addition, capability of phospholipase production is not associated with the site of isolation, as we could show that the mean Pz did not statistically differ between isolates from various sources of infection.

Extracellular proteinase is among the virulence factors produced by *C. albicans*, which has been well recognized for host tissue damage. However, more specific functions of secreted proteinase have been additionally suggested, which are cell wall maintenance and remodeling, formation and resistance of polymicrobial biofilms, adhesion to external protective barriers of the host, deregulation of host proteolytic cascades, interaction with host defense cells and also antibodies, complements system, and antimicrobial peptides production (Rapala *et al*., 2018). Extracellular proteinase is known as secreted aspartic proteases (Saps). There have been 10 *SAP* genes reported in *C. albicans* so far. Sap1-8 are soluble enzymes whereas Sap9-10 are GPI anchors attached to cell wall (Mayer, 2013; Rapala *et al*. 2018).

Surprisingly, we report here a relatively lower incidence of proteinase production in *C. albicans* isolates (29%) compared to several other studies (Alenzi, 2016; Shirkhani, Sepahvand, Mirzaee, & Anbari, 2016; Tay, Abidin, Hassan, & Ng, 2011), which have shown 70 - 100% of isolates having proteinase activity. Under our test conditions, the incubation period is obviously sufficient for production of enzyme and for it to be secreted into agar medium. Amido black dye staining was also used to increase the contrast between agar containing protein and clear zone, reducing possible errors from misinterpretation. ATCC strains were also included in all tests as controls, in our experiments. These suggest that observing less proteinase production among our isolates should be a correct result. Similar to phospholipase, no differences in proteinase production were detected by the source of infection.

Productions of biofilm, phospholipase, and proteinase in *Candida glabrata* and *Candida parapsilosis*, the most common non *albican Candida* (NAC) species, have also shown contradictory findings (Pandey, Gupta, & Tilak, 2018; Silva *et al*., 2012; Tóth *et al*. 2019). It is concluded that these virulence factors are highly strain dependent (Silva *et al*., 2012; Tóth *et al*., 2019). In addition, a network of virulence factors together with host immune responses to infection regulate pathogenesis of candidiasis (Lopes & Lionakis, 2022). Analysis of correlations of the virulence factors seems to provide more conclusive information. In our study, therefore, we analyzed correlations between the biofilm forming ability, phospholipase and proteinase production in *C. albicans*. As most of our tested isolates formed biofilm and produced phospholipase, the positive correlation with statistical significance between these two factors was unsurprising. Similar finding was presented in correlation between adherence to epithelial cells and phospholipase production in *C. parapsilosis* clinical isolates from blood (Dagdeviren, Cerikcioglu, & Karavus, 2005). This study suggests that phospholipase is an important virulence factor in bloodstream infections caused by *C. parapsilosis*. Unfortunately, we could not find such result for *C. albicans*. Taken together, our results suggest that biofilm and phospholipase may synergistically contribute to *Candida* infection. Further investigation in animals experimentally infected by *C. albicans* mutants with biofilm-related and phospholipase genes disrupted is essential.

Although the control group of *C. albicans* isolated from healthy individuals was not included in our study, similar studies to ours, which included *C. albicans* isolates obtained from control group (healthy individuals), revealed comparable outcomes between clinical isolates and control. A comparative study on phospholipase and proteinase production in *C. albicans* isolates obtained from oral cavity of patients with type 2 diabetes mellitus (DM) and controls without type 2 DM showed 100 % of the isolates from both groups capable of phospholipase and proteinase production. Further analysis of Pz revealed no significant difference of phospholipase produced by *C. albican* from non-type 2 DM individuals and from DM patients. However, a difference with statistical significance was observed in proteinase produced by *C. albicans* from Pz value between non-type 2 DM individuals and DM patients (Tsang *et al*, 2007). Another comparative study of biofilm and enzyme production in *C. albicans* isolated from patients and from healthy individuals revealed similar results. Biofilms and phospholipase activity from experimental group and control were not statistically different. Interestingly, proteinase activity of *C. albicans* from healthy controls was higher than in isolates from the patients (Mendes *et al.*, 2023).

C. albicans is a commensal of humans gently living as normal flora over mucosal surfaces of oral cavity, gastrointestinal tract, and vagina. *C. albicans*, however, can shift from harmless commensal to a deadly pathogen upon dysfunction of host immunity or imbalance of endogenous microbiota (Lopes & Lionakis, 2022). Therefore, most of *C. albicans* infections are from endogenous source (Rai, Wijlick, Bougnoux, Bachellier-Bassi, & d'Enfert, 2020). According to several studies related to prevalence of virulence factors in *C. albicans*, it is obvious that capability of forming biofilm and hydrolytic enzyme production are highly variable among *C. albicans* isolates (Mendes *et al.*, 2023; Mohammadi, Charkhchian, & Mirzadeh, 2023). Comparing the production between isolates from different individuals may be inconclusive unless biological matched controls and cases are available. In order to obtain significant information, therefore, consecutive isolates from the same patients collected at disease onset and when infection free might be useful.

In conclusion, several factors may affect expression of virulence factors, such as strain of *Candida* spp., type of infection, stage of infection, and geographical region. To broaden the understanding of epidemiology of the important virulence factors mostly found in *C. albicans*, we showed here that biofilm formation and phospholipase production seem to be more virulent compared to proteinase production in our tested isolates. The results on hydrolytic enzyme activity can

underline the importance of phospholipase as a possible pharmacological target for treatment of *Candida* infections. Inhibitor of phospholipase, therefore, could be an alternative.

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