International Journal of Molecular and Clinical Microbiology 10(2) (2020) 1402-1409

International Journal of
Molecular and Clinical Microbiology

Comparison of biofilm formation and enzymatic activities of different clinical *Candida* species from different parts of Iran

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ARTICLE INFO

Article history:
Received 5 July 2020
Accepted 27 November 2020
Available online 1 December 2020

Keywords:
Enzymatic Activities;
Biofilm formation;
Candidiasis

ABSTRACT

Candidiasis is one of the serious opportunistic fungal infections caused by different species of Candida, especially *Candida albicans*. The extracellular enzymatic activities are determined as principal factors for pathogenesis of *Candida* spp., involved in the degradation of proteins and adhesion during biofilm formation. The study was performed on 100 *Candida* isolates prepared from patients with various forms of Candidiasis, referred to the Laboratory. All isolates were confirmed by phenotypic methods. The evaluation of enzyme activity of different of *Candida* spp. was done in chromogenic media. In addition, the biofilm formation of all isolates was done by MTT method. Our results indicated the proteinase activity was positive (mean 0.015), hemolysin activity was positive (mean= 0.027), phospholipase activity was positive (mean=0.52) and esterase activity was positive (mean=0.003). The highest and lowest biofilm formation were seen in in *C. glabrata* with (OD=0.62) and *C. tropicalis* with (OD=0.46), respectively. The secretion of several enzymes by *Candida* isolates has been identified for their virulence factors in Candidiasis. Our data recommend that biofilm formation is probably to key a role in the pathogenicity of Candidiasis, and in patients with immunocompromised, this might be due to the capability of *C. albicans* to adapt to the changed physiological environmental.

1. Introduction

Candida spp. infections are the main reason of the high morbidity and mortality in the immunocompromised patients (Giri et al., 2012). These diseases range from superficial and mucosal infections to serious systemic infections or even deadly (Pereira et al., 2016). Although *Candida albicans* is the most common *Candida* species responsible for infections in the different clinical forms of Candidiasis, other *Candida* species, such as *C. tropicalis*, *C. glabrata*, *C. krusei*,*C. parapsilosis*, and *C. guilliermondii* are often isolated from patients (Pfaller et al., 2016). Non-albicans species (such as *C. tropicalis* and *C. glabrata*) have become more important in recent years due to their resistance to some antifungal agents (Silva et al., 2011).

Several factors are involved in the pathogenicity and resistance of *Candida* to the immune system (Mayer et al., 2013; Yang et al., 2003). Extracellular hydrolytic enzymes play an important role in *Candida* growth, especially *C. albicans* (Sacristan et al., 2011).These enzymes

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facilitate attachment to tissues and invade the host tissue (El-Houssaini et al., 2019). Phospholipase, proteinase and esterase are the most important enzymes produced by *C. albicans*. The lipolytic enzymes (Phospholipase and esterase) are responsible for lipids degradation for nutrient gaining, adhesion to host cells, and the beginning of inflammatory processes (Pandey et al., 2018; Keyhani, 2018). Aspartyl proteases are responsible for digestion for proteins, innate immune evasion and allowing the opportunistic pathogenic fungus to escape from the first line of cell host defenses (Monika et al., 2017). Hemolysin is critical to obtaining iron from the host's erythrocytes, which is considered one of the vital micronutrients for the opportunistic fungi pathogen to progress and endure in their hosts (Nayak et al., 2013).

Biofilms in *C. albicans* are composed of yeast, hyphae and pseudohyphae covered by extracellular matrix, which form upon adherence to live or non-live surfaces. The biofilm formation is another crucial virulence factors for a number of *Candida* species (El-Houssaini et al., 2019; Ramage et al., 2012). Biofilms are responsible for a wide range of infections and mortality, especially in nosocomial infections (Sardi et al., 2013). The aim of this study was to evaluate the effects of biofilm formation and the enzymatic activities (aspartyl proteinase, esterase, hemolysin and phospholipase) from different clinical *Candida* spp from different areas of Iran during 2018-2019.

2. Materials and Methods

In this cross-sectional study, the isolates were collected from Mazandaran University of Medical Sciences. Totally 100 yeast isolates (Blood, skin, mocous membrane, etc) isolated associated with invasive candidiasis or other forms such mocutaneous, superfacial collected from all over Iran) were examined, including the following species: 39 isolates of *C. albicans*, 44 isolates of *C. krusei*, 16 isolates of *C. tropicalis* and 1 isolates of *C. glabrata.* The isolates of *Candida* sp. were then recognized by sub-culturing on CHRO Magar *Candida* (CHRO Magar, France), production of hyphae /pseudohyphae, germ tube and chlamydospores tests.

2.1. Proteinase assay

Proteinase activity was measured by bovine serum albumin (BSA) as previously by Sachin described. The BSA media contained 0.1% Kh2Po4, 0.05% MgSo4, 0.2% BSA, 1% dextrose and 0.01% yeast extract. The medium pH was reached 5.0 by the addition of 10 µl of hydrochloric acid (Sigma-Aldrich) and sterilized by filtration and then mixed with melted agar until a final concentration of 2% w/v of BSA (Sigma-Aldrich) in agar was attained. Ten microliters of each *Candida* suspension (10^6cells/mL) were stopped and inoculated on the BSA plates. Each plate was incubated for 48 h at 37°C and then the diameter of both colonies and the precipitation zone were measured to calculate the enzymatic activity index (EAI) (Sachin et al., 2012).

2.2. Esterase assay

Esterase activity was measured using the Tween 80 opacity test medium. 10 g Bacto peptone (Sigma-Aldrich), 5 g sodium chloride (NaCl), 0.1 calcium chloride ($CaCl_2$) and 15 g agar were dissolved in 1000ml distilled water. The autoclave was gradually cooled down until the temperature reached about 50°C and then autoclave 5 ml of Tween 80 was added to the media and distributed to 8 cm sterile plates. Ten microliters of each *Candida* suspension (10^6 cells/mL) were inoculated on points of the plate and precipitation zone was measured to calculate the EAI at 30°C (Rajendran et al., 2012).

2.2. Phospholipase assay

Phospholipase activity was measured by the method described by Kantarcio□lu and Yücel. The egg yolk media contained Sabouraud's dextrose agar (SDA) 65 g, Nacl 4.58 g and $Cacl_2$ 5.5g in distilled water 920 ml. 80 ml sterile egg-yolk emulsion, which had been centrifuged at 3,000 × g for 15 min at room temperature was then added to the mixture at 45°C, and then distributed to 9 cm plates. Ten microliters of each *Candida* suspension (10^6cells/mL) were spot inoculated on the

surface of the egg yolk agar. EAI for phospholipase was calculated as mentioned for the other enzymes. Thus, ten microliters of a standardized fungal suspension (10^8cells/mL) were inoculated on blood SDA medium(Merk) with glucose and plates were incubated in a 5 % CO2 incubator at 37°C for 48h. The mean colony diameter (with four replicates per sample) and the transparent halo diameter around each colony were used to calculate the enzymatic activity (Hz) (Kantarcio□lu et al., 2002).

2.3. Haemolysin assay

C. albicans strains haemolytic activities were examined on blood SDA(Merk) plates by the process defined by El-Houssaini et al., 2018 and Manns et al., 1994. Aliquots (5 µL) of standardized yeast inoculum of the formerly mentioned antifungal-treated and control suspensions were spot injected aseptically onto the blood SDA plates, which were left to dry out and then incubated at 37°C for 48 h under 5% CO2. The plates were tested for the presence of haemolysis zone around the colonies, which discloses haemolytic activity. Beta-hemolysin breaks down the red blood cells and hemoglobin completely. This leaves a clear zone around the fungi growth. Such results are referred to as β-hemolysis (beta hemolysis). Alpha-hemolysin partially breaks down the red blood cells and leaves a greenish color behind. This is referred to as α-hemolysis (alpha hemolysis). The haemolytic index (Hz) was considered in terms of the ratio between the diameters of translucent halo and colony (Wu Tn et al.,1996).

2.4. Biofilm formation assay

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. The MTT assay has high-throughput potential and can be efficiently used for determination of biofilm-forming capacity of microorganisms. First, a loop of *Candida* colonies grown on SDA medium(Merk)was cultured in 1 ml of YMBB medium (Sigma-Aldrich) supplemented with 100 Mm glucose and incubated at 37°C for 24 h, and then the cell suspension was prepared. The cell suspension was poured into each well of a plate. The plates were then placed inside a 37 °C incubator for 90 min to allow suspension cells attach to each well of a plate. Afterwards, 200 µl of YNBB medium (Merk)supplemented with glucose was added to each well. The plates were incubated for 48 hours. After the incubation period, the biofilm formed was washed with 100 µl PBS(Phosphate-buffered saline, Sigma-Aldrich).

The yeast cells were seeded in 96-well plates at a density of 1×10^6 cells per well in a 200 µL yeast nitrogen base (YNB) and incubated at 37 °C for 24 h in a medium containing 100 mM glucose. After elapsing time and washing with buffer, MTT (related tetrazolium salts) with YNB supplemented with 100 mM glucose were added to each well and incubation was prolonged for 5 h at 37°C. After the incubation period, 100 µl DMSO (Sigma-Aldrich)was added to each well and incubates at 37 C for 10 min. Plate absorption at 570 nm was then measured using an ELIS Aplate reader (Memmert, Germany) (Paula-Mattiello et al., 2017).The ODc (OD cut-off value) for biofilm formation was evaluated by the following method: mean OD of the yeast-free negative control + 3 × standard deviation (SD) of negative control (El-Houssaini et al., 2017).

2.5. Statistical analysis

The statistical analysis was performed using SPSS software with version 18. There was statistically significant association between the significance level of hemolysin's and esterase's enzymatic activity and colony less than 0.05 mm in diameter. The statistical analysis of MTT results was performed using student's t-test in SPSS.

3. Results

According to Table1, a total of 86 out of 100 *Candida* samples were positive for hemolysin activity (Figure 1) with Concerning to 3 replicates per sample (mean =0.63). A total of86 out of 100 *Candida* samples were positive (Figure 2) for proteinase activity (mean=0.5). Out of 100*Candida* samples, 85 were positive (Figure 2) for phospholipase activity

(mean=0.76). Out of 100 *Candida* samples, 83 were positive (Figure 1) for esterase activity (mean=0.46).

A correlation between the enzymatic activity indexes and different *Candida* spp. were shown in Table 2. Isolates with 3 replicates per sample, the mean colony diameter between the transparent halo diameter around each colony were used to measure the proteinase's enzymatic activity. There were no statistically significant differences between with respect to colony diameter, halo diameter and enzymatic activity (P value > 0.05) (Table 3).

The results of MTT method were used to evaluate biofilm formation of *Candida* spp. The strain with OD value ≥0.5 was considered as positive production of biofilm. Out of the 100 *Candida* samples, 47 were positive. Of these positive samples, 39 were identified as *C. albicans*. In biofilm tests, *C. glabrata* had the highest and *C. tropicalis* had the lowest biofilm formation (Table4).

Table 1. Enzymatic profiles of different *Candida spp*. isolated from Clinical Samples

Candida spp.	Number of the isolates	Phospholipase	Proteinase	Esterase	Hemolysin	
					β	α
C. albicans	39	36	33	37	26	9
C. krusei	43	33	36	28	11	27
C. tropicalis	17	17	16	17	7	4
C. glabrata	1	0	1	1	0	1
Total	100	86	86	83	44	41

Table 2.The mean colony diameter and the transparent halo diameter around each colony of different of *Candida* spp

Enzyme (n = %)	**Species**	**N**	**Mean**
Hemolysin α	*C. albicans*	26	**.45729**
	C. krusei	11	**.50206**
	C. tropicalis	7	**.49904**
	C. glabrata	-	-
Hemolysin β	*C. allbicans*	9	**.45356**
	C. krusei	27	**.46828**
	C. tropicalis	4	**.48000**
	C. glabrata	1	**.35367**
Phospholipase	*C. albicans*	36 (%36)	
	C. krusei	33(%33)	**7.72632**
	C. tropicalis	17(%17)	**8.64480**
	C. glabrata	0(%0)	**.17574**
	Total	100	
Proteinase	*C. albicans*	33(%33)	**.13614**
	C. krusei	36(%36)	**.13619**
	C. tropicalis	16(%16)	**.13545**
	C. glabrata	1(%1)	**.17633**
	Total	100	
Esterase	*C. albicans*	37(%37)	**.15570**
	C. krusei	27(%27)	**.15254**
	C. tropicalis	17(%17)	**.15790**
	C. glabrata	1(%1)	**.15589**
	Total	100	

Table 3. Comparison between the enzymatic activity indices(EAI) expressed by different species of Candida isolated from from Clinical samples

Species and EAI Enzyme	*C. albicans*	*C. krusei*	*C.tropicalis*	*C.glabrata*	P-value
Hemolysin α	0.45729	0.50206	0.49904	-	.023
Hemolysin β	0.45356	0.46828	0.48000	0.35367	.151
Phospholipase	23.17896	25.93440	0.52722	-	.473
Proteinase	0.40844	0.40857	0.40635	0.52900	.165
Esterase	0.46711	0.45763	0.47370	0.46767	.519

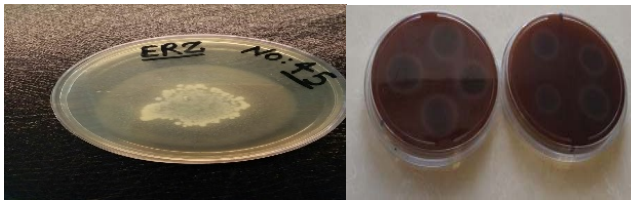

Figure 1. Sediments (precipitates) around the colony for Esterase(left) and hemolytic activity on blood plate assay (right)

Figure 2. Sediments (precipitates) around the colony for Proteinase(left) and phospholipase activity egg yolk agar (right)

Table4. Table of results of ANOVA biofilm analysis

Candida sp.	N	Mean OD	Std. Deviation
C. albicans	39	.50021	.127487
C. krusei	44	.49864	.111380
C. tropicalis	16	.46088	.117709
C. glabrata	1	.62300	.

4. Discussion

Candida yeasts are generally present in healthy humans, frequently part of the human body's normal oral and intestinal flora, and particularly on the skin; however, their growth is normally limited by the human immune system and by competition of other microorganisms, such as bacteria occupying the same locations in the human body (Al-Ahmad et al., 2016).

The most important factors predisposing individuals to Candidiasis in humans are as follows: overuse of antibiotics, prolonged hospital stays, aging, diabetes mellitus, and immunosuppressive drugs used to treat dangerous diseases like cancer (Atalay et al., 2015).

In the study of 100 *Candida* samples, the percentage of enzyme production of Candida spp. was included Phospholipase 86%, Proteinase 86%, Esterase 83%, α Hemolysin 44% and β Hemolysin 41%. According to our study, the highest production of phospholipase, Proteinase, Esterase, alfa Hemolysine and β Hemolysine enzymes, respectively included *C. albicans* 36%, *C. krusei* 33%, *C. albicans* 37%, *C. albicans* 26% and *C. krusei* 27 %.

Dabiri et al (Dabiri et al., 2018) indicated the ability of secretory enzyme formation and biofilm formation in *Candida* spp. isolated from clinical samples. They reported producing proteinase (80.8%)and phospholipase (42.5%) by *Candida* spp. Their results were consistent with our results. The highest production of proteinase, phospholipase and the biofilm formation were seen in *C. albicans*. The results of our study are consistent with their performance.

Sacristán indicated aspartyl proteinase, hemolysin, phospholipase activity and biofilm formation in 16 isolates of *C. albicans* (Sacristan et al., 2011). In the present study, 47%, 58% and 31% of *C. albicans* isolates were positive for biofilm formation, proteinase, andphospholipase production, respectively.

Out of the 100 *Candida* samples, 47 were positive. Of these positive samples, 39 were identified as *C. albicans*. In biofilm tests, *C. glabrata* had the highest and *C. tropicalis* had the lowest biofilm formation (Table4).

In contrast to present study, Sánchez-Vargas et al (Sánchez-Vargas et al., 2013) reported that the oral isolates of *C. glabrata* were strong biofilm production, whereas *C. albicans* and *C. tropicalis* were moderate production. The results of our study are consistent with their performance.

Tee et al (Tay et al., 2011) evaluated proteinase, phospholipase, and biofilm production in *Candida* spp. Isolated from blood cultures of Malaysian patients. There was no significant difference in the expression levels of proteinase and biofilm production amongst the *Candida* isolates, but the phospholipase activity of *C. albicans* was significantly higher than that of the non-albicans *Candida* spp. The results of our study are consistent with their performance.

Pakshir et al (Pakshir et al., 2013) reported 98.5%, 4.5% and 82.1% of *Candida* isolates showed hemolysin, coagulase activity and biofilm formation, respectively. There were no significant difference was seen between of *Candida* isolates with enzyme and biofilm production. The results of our study are consistent with their performance.

Paula et al (Paula-Mattiello, 2017) 22 isolates of *C. parapsilosis* that produced proteinase and 3 isolates secreted phospholipase. In addition, they were seen biofilm formation in all isolates. The results of our study are consistent with their performance.

In Sachin et al (Sachin et al., 2012) study, the phospholipase assay was determined in 60.9% of strains, 59.1% produced proteinase and haemolysin assay was demonstrated seen in 51.8% of *Candida*strains. The maximum extracellular hydrolytic enzymes production was seen in *C. albicans* and the highest phospholipase and proteinase activity was belonged to *C. tropicalis* whereas, haemolysin activity was more in *C. dubliniensis*. Non-albicans isolates activity results were inconsistent with our results.

Maheronnaghsh et al (Maheronnaghsh et al., 2019) reported the mean phospholipase activity of *Candida* isolates was 0.795 and 0.775, proteinase activity was 0.7531 and 0.7558, esterase activity was 0.6142 and 0.7186, and hemolysin activity was 0.6317 and 0.5756, respectively.

In Pandey et al (Pandey et al., 2018) study, *C. albicans* (89.86%) was the main proteinase production, while 95.8% of *C. tropicalis* induced hemolysis. Moreover, they were no

funded any esterase activity in *C. krusei* and *C. glabrata* isolates.

Gokce et al (Gokce et al., 2007) indicated *C.albicans* isolates were identified as proteinase positive whereas 8 (25.8%) non-albicans isolates were proteinase positive ($P < 0.05$).

In addition, the phospholipase activity was positive in 41 isolates of *C. albicans*, while all non-albicans isolates were negative. In line to present study, Treviño-Rangel et al (2013) identified hemolysis activity between the strains studied. In present study, the proteinase had the highest enzymatic activity and hemolysin had the lowest enzymatic activity in all strains. The difference in results is due to differences in the type of assay, number of isolates, type of strain, source of infection and geographical distance.

Conclusion

These outcomes recommend that the high adherence capability of isolates participates in initial biofilm formation. The relation of adherence ability to cells with biofilm formation can be a significant parameter to distinguish invasive from non-invasive isolates.

Refereces

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