

Virulence Factors of Yeasts Isolated From Oral Lesions

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ABSTRACT

Objectives: To detect the virulence factors of yeasts which are proteinase and phospholipase which have important role in the pathogenesis of oral lesions.

Patients and methods: This study included 120 children manifested with oral lesions admitted to the Consulting Clinic and Medical Wards of Ibn – Sina and Ibn Al – Ather Teaching Hospitals in addition to Neonatal Care Unit of Al – Battol Teaching Hospital. Samples collected during the period from December 2012 – May 2013. Control group was 60 apparently healthy children from whom oral swabs 'were obtained. Identification' of the isolated yeasts were conceded by "direct examination, culture, biochemical tests and API – 20 C system, then detection of' protienase and phospholipase of yeasts isolated from oral lesions".

Results: *Candida albicans* isolates have a high expression rate for the production of proteinase (95.3%) and phospholipase(97.1%) in comparison to the other candida and yeast species with a significant difference ($p = 0.001$).

Conclusion: The isolates produce two important virulence factors namely proteinase and phospholipase significantly more in *Candida albicans* than other yeasts.

Keyword: Virulence factors, candida species, oral lesions.

عوامل الفوعة للخمائر المعزولة من آفات الفم

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الخلاصة

الهدف من الدراسة: الكشف عن عوامل الفوعة للخمائر وهي إنزيم البروتيناز وإنزيم الفسفوليباز ممن لهم دور مهم في سير مرض آفات الفم.

عينات الدراسة وطرائق العمل: تضمنت الدراسة 120 طفل ظهرت عليه آفات الفم والذين يراجعون العيادة الاستشارية والردهات في مستشفيات ابن سينا و ابن الأثير التعليمية فضلا عن وحدة الحاضنة في مستشفى البتول التعليمي. تم جمع العينات السريرية للفترة من كانون الأول 2012 لغاية أيار 2013. شملت العينات السريرية مسحات من آفات الفم. كما تضمنت الدراسة مجموعة السيطرة التي شملت 60 من الأطفال الأصحاء وتم الحصول منهم على 60 مسحة فموية. تم تمييز الخمائر المعزولة بواسطة الفحص المباشر، الزرع، الفحوصات الكيمحيوية واستخدام نظام API – 20 C ومن ثم فحص إنزيم البروتيناز والفسفوليباز للمبيضات المعزولة من آفات الفم.

النتائج: أظهرت النتائج بأن *C.albicans* أعطت أعلى نسبة معبرة في إنتاج إنزيم البروتيناز(95.3%) والفسفوليباز(97.1) بالمقارنة مع المبيضات وأجناس الخمائر الأخرى مع اختلاف ملحوظ بينهم($p = 0.001$).

الاستنتاج: العزلات أنتجت نوعين من عوامل الفوعة وهي إنزيم البروتيناز والفسفوليباز وهي الأكثر دلالة في عزلات *C. albicans* عن الخمائر الأخرى.

INTRODUCTION

Candida species are commonly in attendance in the oral flora as a parasite as well as in nature as saprophyte¹. beneath confident physiological and pathological situation, the yeasts may modify status from that of commensal to a pathogen predominantly in debilitated patient². Oral candidiasis is a warning of impaired local or systemic defense mechanisms. The chief restricted predisposing factors are concentrated saliva secretion, mucosal lesions, decreased blood distribution in the mucosa due to radiation therapy³. The general predisposing factors are diabetes mellitus, chronic illnesses⁴, acquired or inborn immunodeficiency, malignancies, and malnutrition⁵. The iatrogenic factors like wide spectrum antibiotics, corticosteroid therapy chemotherapy and local trauma⁶.

The development of oral candidiasis is the result of imbalance between fungal virulence factors and host's defense². Fungal virulence factors are essential for the conversion from colonization to illness. Extracellular hydrolytic enzymes seem to play an important role in candida overgrowth as these enzymes facilitate adherence and tissue penetration⁷. Among the the majority significant hydrolytic enzymes produced by candida are phospholipase and proteinase which play a important role in pathogenesis⁸. The secreted phospholipase and proteinase are not limited to *Candida albicans* only but their attendance have been demonstrated in other non-*Candida albicans* species as *C. glabrata* and *C. tropicalis*⁹.

PATIENTS AND METHODS

Patients: One hundred and twenty children manifested with oral lesions characterized by the presence of removable creamy white patches or red atrophic patches in the mouth were enrolled in the study. Males were 59(49.2%) and females were 61(50.8%). The age of the studied children ranged from 2 days – 12 years.

Control group: consist of 60 apparently healthy children. They were 29(48.3%) males and 31(51.7%) females. Their ages ranged between 3 days –12 years. Sex and age were matched with the patients group.

All samples were collected from the children admitted to the Consulting Clinic and Medical Wards of Ibn – Sina and Ibn - Al – Ather Teaching Hospitals in Mosul in addition to the Neonatal Care Unit of Al – Batool Teaching Hospital in Mosul. The clinical specimens were collect during the period from December 2012 – May 2013.

Processing of the clinical specimens

"Swabs were cultured on Sabouraud dextrose agar media and brain–heart infusion blood agar media for primary isolation. Direct examinations were done by Gram stain and 20% KOH mount. Positive culture underwent further identification as germ tube production, chlamyospore formation, and API – 20 C system tests. Final test of virulence factors detection including phospholipase and proteinase was done".

Detection of virulence factors:

1 - Phospholipase

Extracellular phospholipase activity was detected by inoculation of 10 µl of the yeast suspension and incubated at 37°C intended for 4 days.^{10, 11} "Phospholipase activity (Pz) was measured by dividing the colony diameter (Dc) over colony diameter plus precipitation zone diameter (Dz) by: $Pz = Dc / Dc + Dz$.^{1, 12} The results were subdivided into four categories : Pz = 1 (Negative); Pz = 0.9 - 1 (+); Pz = 0.89 - 0.80 (++) ; Pz = 0.79 - 0.70 (+++); Pz ≤ 0.69 (++++). Accordingly, a low Pz value indicates stronger enzyme activity".^{1, 13, 14, 15}

2 - Proteinase

The extracellular activity of secreted aspartyl proteinase was detected by a small portion of isolated yeast colony (grown on Sabouraud dextrose agar for 48 hours) was suspended in yeast extract broth, 10 µl of the suspension was placed in a well punched on the surface of bovine serum albumin agar. The inoculated plate was

incubated at 37°C then checked for opacity around the well up to 3 days. "The proteinase activity (Pz) is defined at the ratio of colony diameter (Dc) and colony diameter plus degradation zone diameter (Dc + Dz) by: $Pz = Dc / Dc + Dz$.¹⁴

The low Pz the high production of the enzyme. The scoring was carried out by determination of the proteinase zone value as for phospholipase¹³.

Statistical analysis

The data were analyzed statistically by using tables, pie, and bar charts according to¹⁶:

1. Standard statistical methods were used to describe the results of: (mean, standard deviation (SD), number, and percentage).
2. Unpaired t – test.
3. Proportion test and Fisher's exact test were used to find out the relationship between isolated *C.albicans* and other species from oral samples and from other sources; they were used to find out the relationship between proteinase and phospholipase.
4. The statistical results were considered significant at $p \leq 0.05$.

RESULTS

Yeast Species	Virulence Factors			
	Proteinase		Phospholipase	
	No.	%	No.	%
<i>C. albicans</i>	81	95.3	67	97.1
Other species	4	4.7	2	2.9
Total	85	100	69	100

Table 1: Percentage of the virulence factors produced by *Candida albicans* and other species isolated from oral lesions.

A significant difference between *C. albicans* and other yeast species in the ability of production of virulence factors at $p=0.001$ using Proportions and Fisher's exact test.

Table 2: Types of yeast species isolated from oral lesions with scoring of their virulence factors.

Yeast species	Total No.	Virulence Factors	
		Proteinase	Phospholipase
<i>C. albicans</i>	92	11 (-)	25 (-)
		16 (+)	14 (+)
		8 (++)	11 (++)
		10 (+++)	13 (+++)
		47 (++++)	29 (++++)
<i>C. tropicalis</i>	2	1 (++)	-
<i>C. parapsilosis</i>	2	-	-
<i>C. guilliermondii</i>	1	++++	-
<i>C. luistaniae</i>	1	++++	-
<i>Cr. Laurentii</i>	4	1 (++)	-
<i>R. rubra</i>	2	-	1 (+)
			1 (+++)
<i>S. cerevisiae</i>	1	-	-
<i>T. asahii</i>	1	-	-
Total	106	85 (80.2%)	69 (65.1%)

A significant difference between proteinase and phospholipase using Proportions test and Fisher's exact tests ($p = 0.012$).

Table 3: Candida species isolated from control group concerning virulence factors.

Yeast Species	Total No.	Virulence Factors	
		Proteinase	Phospholipase
		5 (-)	10 (-)
<i>C. albicans</i>	18	5 (+)	5 (+)
		8 (++)	3 (++)
<i>C. guilliermondii</i>	2	-	-
<i>C. parapsilosis</i>	1	++	-
<i>C. tropicalis</i>	1	-	-
<i>C. lusitanae</i>	1	-	-
Total	23	14 (60.9%)	8 (34.8%)

No significant difference between proteinase and phospholipase according to proportions test and Fisher's exact test ($p = 0.067$).

1. Proteinase

a. Scoring of yeasts' proteinase isolated from oral lesions

Out of the 106 isolated yeasts, 85(80.2%) produced proteinase enzymes with different scoring. From the 92 isolates of *C. albicans*, 81 isolates produced proteinase, though 47 isolates of them showed a stronger activity of proteinase production with 4 + scores, followed by (10) isolates with 3 + scores, (8) with 2 +, and (16) with only 1 + scoring. The other 4 candida species showed different scoring (Table 8). On the other hand, the other 8 isolated yeasts species, only one isolate of *Cr. laurentii* showed 2 + score for the production of proteinase enzyme Table 2 and Figure 1 .

b. Scoring of yeasts' proteinase isolated from control group

Out of 23 isolates of 5 candida species, only 14(60.9%) produced proteinase. Thirteen were *C. albicans*. Concerning scoring eight isolates had enzymatic activity of (++) and only 5 with (+)

score. The last one *C. parapsilosis* had an enzymatic activity of (++) scoring Table 3 .

2. Phospholipase

a. Scoring of yeasts' phospholipase isolated from oral lesions

Out of 106 yeast isolates, only 69(65.1%) produced phospholipase. Only *C. albicans* produced the enzyme. The 67 isolates of *C. albicans* produced the enzyme with different scoring that (29) isolates with an enzymatic activity of 4 +, followed by 13 (3 +), 11 (2 +), and 14 (+). Among the 4 species of the other yeasts, only 2 isolates of *R. rubra* showed only one score of enzymatic activity and only 1 with (+++), Table 2 and Figure 2.

b. Scoring of yeasts' phospholipase isolated from control group

Phospholipase enzyme was produced from 8 isolates of *C. albicans* (34.8%) out of 18. Concerning enzymatic scoring, 3 isolates with (++) and only 5 with (+). The others *Candida* species did not produce the enzyme Table 3 .

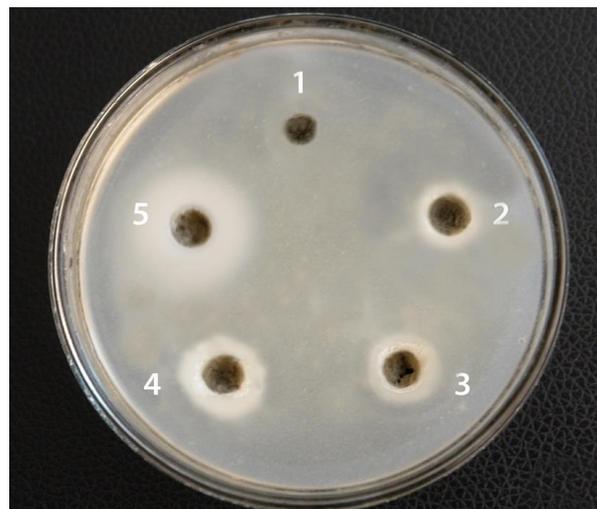


Figure 1: The scoring of proteinase enzyme produced by yeasts on bovine serum albumin medium

- 1. negative
- 2. + = low active
- 3. ++ = moderate active
- 4. +++ = active
- 5. ++++ = highly active

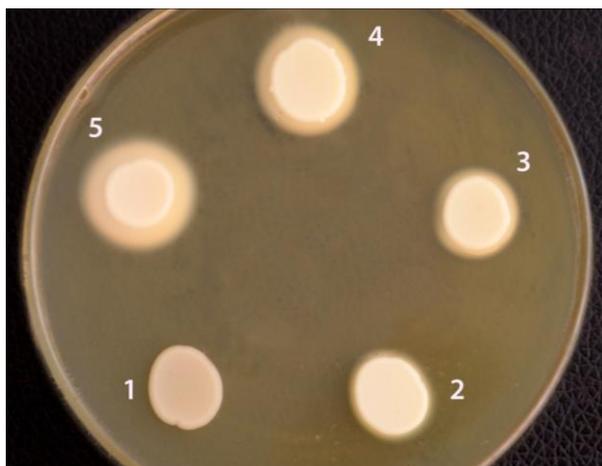


Figure (2): The scoring of phospholipase enzyme produced by yeasts on egg yolk medium

1. negative
2. +=low active
3. ++=moderate active
4. +++=active
5. ++++ = highly active

DISCUSSION

Virulence Factors

Fungi are able to cause a disease and to overwhelm the host defense system because of possessing several genes and proteins which are associated with the pathogenicity; they are called virulence factors.¹⁷

Extracellular secreted enzymes of microbial pathogens have gained considerable attention due to their potential roles in pathogenesis and as possible targets for future antimicrobial therapies.¹⁸

Kantarcioğlu and Yuçel (2002) reported that the most significant hydrolytic enzymes formed by candida are proteinase and phospholipase which play a major role in pathogenesis.¹⁹ The role of these two hydrolytic enzymes in *C. albicans* and other yeast species seem to be related to species virulence.²⁰

1. Proteinase Activity

In the present study, the isolates from the oral lesions that produced proteinase enzymes with different scoring were *C. albicans*, *C. tropicalis*, *C. guilliermondii*, and *C. luistanae* where as *C. parapsilosis* did not produced such enzyme. On the other hand, concerning control group, *C. albicans* and *C. parapsilosis* were produced the enzyme, while *C. guilliermondii*, *C. tropicalis*, and

C. luistanae did not. Furthermore, other investigators mentioned that *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. guilliermondii*, and *C. luistanae* were isolated in their study from control group and produced proteinase enzyme.²¹

In this current study, out of the 92 *C. albicans* isolates, 81 produced proteinase enzyme from this the higher activity of proteinase was recorded mainly for *C. albicans* (95.3%) isolated from oral thrush in comparison with control group. This is confirmed by previous study where 26 (66.6%) isolates of *C. albicans* exhibited strong proteinase activity detected from clinical sources (urine and saliva) in comparison with 4 (13.3%) isolates from throat swabs of the normal healthy control. Tsang and Colleagues (2007) reported that proteinase creation from the diseased patients when compared to healthy individuals was significantly larger in *C. albicans* isolated.²²

In this study, the greater part of *C. albicans* enzyme producers were measured to have very physically powerful activity. From the 81 isolates of *C. albicans* with positive proteinases activity, 47 showed the highest activity with scoring of (4 +) as (proteinases activity zone), followed by 10 isolates with scoring of (3 +), 8 with (2 +) and 16 with only (+) activity (Table 2). Oksuz and Colleagues (2007) reported different frequencies of proteinase activity ranged from (4 +) to (1 +) but the highest value within the range of 3 +.²¹ There was numerous reports representative invasive strains of *C. albicans* produced extensively more extracellular enzymes activity than the commensal strains do.^{10, 23}

Regarding the 4 NCAC species that were isolated during the study from oral lesions, each of *C. guilliermondii* and *C. luistanae* showed high activity of proteinase enzyme (4+), one out of 2 isolates of *C. tropicalis* showed 2 + scoring, while the 2 isolates of *C. parapsilosis* did not show any activity. The secreted aspartyl proteinases are not restricted to *C. albicans* and their presence has been confirmed in *C. tropicalis*, *C. parapsilosis*, and *C. guilliermondii*.⁹ In recent years, species of candida other than *C. albicans*, which were in the past considered as a fewer or non - virulent, have been occupied more frequently in human sickness.¹⁵

Kantarcioğlu and Yücel (2002) reported that protease activity was observed in addition to *C. albicans*, in *C. kefyr*, *C. parapsilosis*, and *C. tropicalis* isolates.¹⁹ Protease activity of *C. albicans* isolates were established to be (56.7%) elevated more than that of NCAC isolates (43.9%) but the variation was not statistically significant.²¹ These results were not in agreement with this of the current study that the activity of *C. albicans* (95.3%) was statistically different from NCAC (4.7%) in the production of proteinase enzymes.

Concerning the other yeast species, none of which showed proteinase activity except one out of the 4 isolates of *Cryptococcus laurentii* which showed an activity of (2+) scoring. It was reported that cryptococcal infection due to species other than *Cryptococcus neoformans* may be considered an emerging infection, and proteinase secretion was a virulence factor of *Cr. neoformans*.²⁴ *Cryptococcus laurentii* was not reported as producer of proteinase enzyme when isolated from clinical specimens. Moreover, it was mentioned that none of 38 *Cr. laurentii* isolates that are identified from pigeon droppings and surrounding hospital areas exhibited proteinase activity.²⁵

2. Phospholipase Activity

Out of 106 studied yeast species, phospholipase activity was noticed only in 69 (65.1%) isolates. Kantarcioğlu and Yücel (2002) report more constructive rate of phospholipase activity from patients with invasive yeasts disease. In this study, it was appeared that proteinase activity (80.2%) was higher than this of phospholipase (65.1%) from the yeasts with a significant difference ($p=0.012$). This is in consistence with other report that 50 of the isolated yeast species was phospholipase positive and 64 was protease positive.

In this study, from the 92 *C. albicans* isolates, only 67 produced phospholipase enzyme with different activities, while no other candida species produced this enzyme. Ghannoum (2000) reported that only *C. albicans* was capable to create phospholipase⁹. Now – a day, it is known that the other species of candida also create the enzyme usually in smaller amount.²⁶ The enzymatic activity was more distinct in *C. albicans* among 100% phospholipase, in contrast to NCAC species which produced only 29.6% of phospholipase.¹³

Pinto and Coworkers (2008) reported 99.4% isolates of *C. albicans* with phospholipase activity.²⁶ However, other researchers state that other candida species such as *C. glabrata*, *C. guilliermondii*, and *C. tropicalis* secreted smaller amount of phospholipase.^{14, 21} Moreover, a higher frequency of *C. albicans* (92.3%) produced phospholipase.¹⁵

In comparison to the control group, phospholipase produced by only 8 isolates of *C. albicans* out of 23. This result is in consistence with this by Basu and Coworkers (2003) who reported that 49% were clinical isolates of *C. albicans* confirmed phospholipase activity while only two since the normal healthy personnel were positive for this enzyme.²⁷ There were more than a few reports indicating invasive strains of *C. albicans* produced extensively additional extracellular enzymes activity than the commensal strain do.^{10, 27} Hence, the levels of phospholipase and protease activities in commensal isolates was found to be lesser than the level reported in clinical *Candida* species.²³

As shown in Table 2, the highest amount of phospholipase enzyme was produced from 29 out of 67 *C. albicans* isolates with 4+ scoring, followed by 13 isolates with 3+ scoring. Mahmouadaabadi and Coworkers (2010) reported that 61 out of 72 yeast isolates produced large amount of the enzyme with score of 4+, and 7 isolates with 3+ score.²⁸ Special frequencies of phospholipase have been reported from different candida species from diverse different sites.^{19, 29}

In a study conducted by Vidotto and Coworkers (1999b) phospholipase activity was found most frequently in oral cavity isolates.³⁰ An elevated phospholipase production is associated with an improved capability to adherence and a superior death rate in animal models.³¹

In the present study, only the 2 isolated yeasts of *R. rubra* showed phospholipase activity while the other isolated yeasts showed no such activity. This result in agree with that of Mayser and Coworkers (1996) who showed that in addition to *C. albicans*, a total of 110 strains of 16 other yeast species were investigated for possible phospholipase production, only *R. rubra* showed the enzyme activity.³¹ *Rhodotorula rubra* has infrequently been confirmed as a pathogen in human. It was believed that factor such as the

condensed growth at 37°C, lack of dimorphism and little capability to adherence reduce important of elevated phospholipase activity in *R. rubra* as a pathogenicity determinant.³² In the current study, neither *S. cerevisiae* nor *T. asahii* produced phospholipase or proteinase enzymes which is in agreement with Dag and Cerikeioglu (2006) who reported that non of the 48 strains of *S.cerevisiae* and *T . asahii* showed phospholipase nor proteinase activity, while all were esterase producer.³³

Colombo and Coworkers (2011) reported that *Trichosporon* species be able to signify the most second or third frequent non –candida yeast infection cause persistent diseases in patients³⁴, but little reports have address the virulence factor of this species.^{35,36}

CONCLUSION

Higher frequencies with different scorings of proteinase (80.2%) and phospholipase (65.1%) are detected in the yeast isolated from oral lesions in children compared to healthy control group.

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