Available online at https://www.sjomr.org.in

SCIENTIFIC JOURNAL
OF MEDICAL RESEARCH

Vol. 7, Issue 27, pp 9-14 , 2023

ISSN: 2520-5234

RESEARCH ARTICLE

Evaluation of Virulence Factors of Yeasts Isolated from Cancer Patients and their Surrounding Environment

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ARTICLE INFORMATIONS

Article history:

Received: 18 July 2023

Revised: 26 August 2023

Accepted: 29 August 2023

Published: 24 September 2023

Keywords:

Biofilm, Hydrolytic enzyme, Virulence factors, Yeasts.

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ABSTRACT

Objective: This study included the determination of virulence factors for yeasts isolated from cancer patients and their surrounding environment through the study of extracellular hydrolytic enzymes and biofilms.

Methods: Using particular media for enzymes, there were three enzymes used, phospholipase, proteinase, and hemolysin, to examine the enzyme activity of yeasts isolated during the study. The microtiter plate method was also used for the purpose of biofilm detection.

Results: The results showed the activity of yeasts towards enzymes as well as biofilm. The largest percentage of yeasts showed proteinase and hemolysin activity. It reached 80 (78.43%) and 79 (77.45%), respectively, while phospholipase was the lowest percentage 49(48.03%). Also, the isolates showed their effectiveness in biofilm formation, as 73 (71.56%) isolates produced biofilm while 29 (28.43%) did not.

Conclusions: The present work concluded that the yeast isolates collected from cancer patients and the environment surrounding them, the great majority of them possess enzymatic activity also have the ability to form biofilms, and this matter is worrying and dangerous for theme because of their presence in the environments of immunocompromised patients and their chance of infection with these yeasts were exactly existent.

INTRODUCTION

Biofilm, pigments, and extracellular hydrolytic enzymes are examples of the virulence factors found in yeast cells that increase pathogenicity. These elements improve the pathogen's capacity to survive, penetrate and spread to other organs.[1] Fungi secrete enzymes that break down a wide range of organic materials, and some of them also create biologically active secondary metabolites that encourage their emergence in carbon-rich environments.[2]

Hydrolytic enzyme

The secretion of hydrolytic enzymes, which is known to be important for the pathogenicity of bacteria, protozoa, and pathogenic yeasts, is one factor that contributes to the virulence process. The hemolytic ability, aspartyl proteinases (Sap), phospholipase, and lipases are all crucial components of germ tubes and hyphae. The extracellular hydrolytic enzymes that *Candida albicans* secretes are of utmost importance.[3,4]

CITATION: Al-Darraji ZDG, Ameen MKM. Evaluation of Virulence Factors of Yeasts Isolated from Cancer Patients and their Surrounding Environment. Sci. J. Med. Res. 2023;7(27):9-14. DOI: 10.37623/sjomr.v07i27.02

The aspartyl proteinases (Sap)

The aspartyl proteinases are represented by ten genes in the SAP gene family. While Sap9 and Sap10 remain affixed to the surface of the cell, Sap1–8 are secreted and discharged into the environment. In a mouse model of systemic infection, Sap1-3 has been shown to be necessary for virulence. Saps, on the other hand, contributes modestly yet significantly to *Candida*. It is controversial whether *C. albicans* is pathogenic.

It has received the most in-depth research as one of *C. albicans'* primary virulence factors. Despite the fact that a variety of hydrolytic enzymes are present in many bacteria, proteinases are most frequently associated with virulence.[5,6] Many pathogenic *Candida* spp., including *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis*, have been shown to contain SAP genes, which generate active extracellular proteinases *in-vitro* (*C. tropicalis* is expected to have four SAP genes, whereas *C. parapsilosis* is thought to have at least two). Extracellular proteinases are produced by *Candida* spp. other than *C. albicans*. There hasn't been much discussion in the literature about the significance of Sap proteinase in *C. dubliniensis* pathogenicity. Nonetheless, proteinase synthesis is extremely likely to add to the fungus' pathogenicity, given that *C. dubliniensis* is estimated to have at least nine SAP genes. [2,7]

Phospholipases

Phospholipases are a diverse group of enzymes that break down the ester bonds in glycerophospholipids, the primary building blocks of cell membranes and lung surfactants. Each glycerophospholipid contains a glycerol backbone with one (lysophospholipid) or two (phospholipid) ester connections to hydrophobic fatty acid chains and a third ester linkage to a phosphate head group. The phosphate head group is linked to a number of polar moieties, such as choline, ethanolamine, serine, and inositol, via an ester bond. Phospholipases are categorized into five groups: A1, A2, B, C, and D, depending on which ester bond is dissolved. Two major phospholipases associated with pathogenicity in fungi are phosphatidylinositol (PI)-preferring phospholipase C (PI-PLC/Plc) and phospholipase B (Plb). [2,8]

Hemolysin

Hemolysin enables *Candida* fungi to get iron from host tissues, which they then use for metabolism, development, and invasion during host infection. Iron is necessary for almost all species, whether unicellular or multicellular. Human hemoglobin, among other proteins, contains iron (a component of erythrocytes). The first description of *C. albicans'* ability to utilize hemoglobin as an iron source was made by Moors *et al.*[9]. These findings suggest that binding to erythrocytes via complement system receptors is the first step in *C. albicans* infection in vivo. The erythrocyte then lyses as a result of the hemolysis factor that *C. albicans* produces. This component is a mannoprotein connected to the fungus' cell surface. Yet, it is unclear how *Candida albicans* causes hemolysis and what the underlying molecules are.[10]

Biofilm

A biofilm is a structure of adhering bacteria wrapped in a self-produced extracellular polysaccharides matrix on any biotic or abiotic moist surface. It may consist of one or several species. In addition to polysaccharide, which makes up the majority of the biofilm matrix, other substances like proteins, phosphorus, hexamines, and uronic acid also contribute to biofilm development depending on the host species and environment.[11,12] One of *Candida*'s main pathogenicity factors is its ability to adapt to a variety of different settings and the formation of surface-attached microbial communities known as biofilms. Both natural host surfaces and the biomaterials used in medical equipment can develop *Candida* biofilms. Such biofilms are noteworthy because they exhibit higher resilience to host defenses and administered antimicrobial medicines than their 'free-living' or planktonic counterparts. Current projections highlight the significance of biofilms.[2] The species of the engaged *Candida* with morphogenesis, qualities of the environment, and other factors all have an impact on the creation and structure of biofilm. The biomaterial to which *Candida* clings and the environment around it.[11,13] *Candida* species benefit greatly from the formation of biofilms, which include protection for their growth and life, increasing symbiotic relationships to enable survival in unfavorable environments and lower susceptibility to antifungal drugs.[14]

Candida species' capacity to create biofilms, which are extraordinarily organized biological and functional communities, is a significant aspect in how they contribute to human disease.

MATERIALS AND METHODS

Yeast isolates

102 yeast were isolated from clinical (skin swabs from cancer patients, control) and environmental (beds, tables, air) samples. These isolates were obtained from the Children's Specialized Teaching Hospital and from the Oncology Center at Al-Sadr Teaching Hospital in Basrah and identified by conventional and molecular methods in our previous study.[15] They include *C. albicans* (25 isolates), *C. krusei* (17 isolates), *C. dubliniensis* (15 isolates), *C. glabrata* (14 isolates), *C. tropicalis* (4 isolates), *C. kefyr* (5 isolates), *Naganishia diffluens* (6 isolates), *N. albida* (5 isolates), *Rhodotorula mucilaginosa* (2 isolates), *Trichosporon asahii* (1 isolate), *Cutaneotrichosporon jirovecii* (1 isolate), *C. orthopsilosis* (1 isolate), *Meyerozyma guilliermondii* (1 isolate).

Enzymatic activities

To test if the yeast isolates could release protease, phospholipase and hemolysin, they were first activated by sub cultured onto SDA at 37°C for 24 to 48 hours.

Elaboration of yeast inoculum

The suspension of activated yeast isolates was prepared by adding small portions of yeast colony to 5 mL of sterile normal saline, then the concentration of cells was adjusted with McFarland scale to get suitable concentration for each test.

- **Proteinase activity:** Proteinase production ability of yeast isolates were measured using bovine serum albumin (BSA) based on Aoki *et al.* In 10 µL of 1×10^6 CFU/ ml from

the suspension of each strain was inoculated as a spot on the center of BSA plates as triplicates. The diameter of the clear zones around the colonies was measured after 7 days of incubation at 37°C as a measure of proteinase production.[16] The presence of proteinase was detected as a translucent zone around the yeast colony. The proteinase activity (Prz) was assessed and computed using Price's method which is described as the ratio of the diameter of the colonies to the diameter of overall colonies plus clear zones. When Prz = 1, no proteinase activity (negative); Prz= 0.7-0.99, weak positive; Prz= 0.5 - 0.69, moderate positive and Prz >0.5, strong positive positive.[17]

- **Phospholipase activity:** Yeasts isolates were tested for extracellular phospholipase activity by measuring the size of the precipitation zone following growth on egg yolk agar according to Samaranayake *et al.* The test and control yeast isolates were inoculated (5 µL of 1× 10^8 CFU/mL) onto the egg yolk agar medium and allowed to dry at room temperature. In 5 mL of saline was added to the plate as control plate.[18] The diameter of the precipitation zone around the colony (an indication of phospholipase activity) was measured after incubation at 37°C for 48 hours. Phospholipase activity (Pz value) was calculated as the ratio of colony diameter to total colony diameter plus precipitation zones and graded as described above.[17]
- **Hemolysis Activity:** According to Luo *et al.*[19], hemolysin activity was measured using SDA supplement with 7 ml fresh sheep blood added to 100 ml SDA enriched with glucose at a final concentration of 3% (w/v). The suspension of yeast isolates was prepared in normal saline to reach to (1×10^8 CFU/mL), after that 10 µL for each isolate was transferred to the center of medium with duplication for isolates in addition to control treatment. All culture media were incubated for 48 hours at 37ºC in 5% CO_2. The appearance of a clear transparent halo surrounding the inoculum site after incubation period showed good hemolytic activity.[18] The hemolytic index (Hz value) was calculated by dividing the colony diameter by the entire diameter of the colony plus the transparent halo. It represents the intensity of hemolysin production by various yeast isolates (no hemolysin activity (negative) when Hz = 1; weak positive when Hz = 0.7 - 0.99; moderate positive when Hz =0.5 - 0.69; strong positive when Hz < 0.5).[17]

Biofilm formation test

To determine the ability of biofilm formation for yeast isolates, used the Microtiter plate method (Marak and Dhanashree)[20] with some modifications. Biofilm formation was performed on a sterile 96-well microtiter plate as described in the following:

Prepare a suspension from an overnight culture on SDA in sterile DW and adjusted it to 1 McFarland for each yeast isolate. SDB is considered a negative control. In 180 µL of Sabouraud dextrose broth supplemented with 1% glucose, each well of the microplate was filled. Then, inoculate 20 µL of the standard suspension of tested isolates. Microplates were covered and incubated at 37°C for 24 hours. After the completion of incubation, microtiter plates were emptied, rinsed with distilled water three times, and then inverted to blot. After that, each well was then filled with 200 µl of 1% crystal violet and incubated for 15 minutes at 37℃. After incubation, the microplates were again rinsed three times with distilled water, then 200 µl of ethanol: acetone mixture (80: 20 w/v) was added to each well. Finally, the optical density (OD) results were read using an ELISA reader, at 450nm, sterile SDB without microorganisms was used as the negative control. The cut-off value was estimated by arithmetically averaging the OD of the wells containing sterile SDB and by adding a standard deviation of +2. Specimens with an OD higher than the cut-off value were considered positive, whereas those with a lower optical density than the cut-off were considered negative.

RESULTS AND DISCUSSION

The proteolytic activity of the extracellular enzymes plays the main role in *Candida* pathogenicity. It is produced by a family of ten secreted aspartyl proteinases (Sap proteins), which do basic functions like breaking down host cell membranes to aid in adhesion and tissue invasion, breaking down or distorting host cell membranes to get nutrients, and breaking down immune system cells and molecules to prevent or combat off antimicrobial attacks from the host.[21] According to Arslan *et al.* [22], it is crucial for *Candida* spp. to degrade a number of host defense proteins, including immunoglobulins, complements, and cytokines, in order to colonize and enter host tissues while evading the host immune system. The obtained findings revealed that the proteinase activity was observed in 80 (78.43%) yeast isolates, whereas 46 (45.9%) of the yeast isolates exhibited high protease activity, 27 (26.47%) isolates had moderate proteinase activity, and 7 (6.86%) isolates showed weak proteinase activity. While 22 (21.56%) isolates were recorded as negative for proteinase activity (Table 1). These findings agreed with previous studies.[5,23-25] They found that *C. albicans* isolated from clinical samples exhibited

Table 1: Proteinase Activity of Yeast Isolates

Yeast isolates (NO)	*Negative*	*Positive*		
		Weak	*Moderate*	*Strong*
C. albicans (25)	5 (20.0%)	4 (16.0%)	2 (8.0 %)	14(56.0%)
C. krusei (17)	2 (11.76%)	2 (11.76%)	9 (52.94%)	4 (23.52%)
C. dubliniensis (15)	3 (20.0%)	0	2 (13.33%)	10 (66.66%)
C. glabrata (14)	2 (14.28%)	0	3 (21.42%)	9 (64.28%)
C. tropicalis (4)	1 (25.0%)	0	2 (50.0%)	1 (25.0%)
C. kefyr (5)	1 (20.0%)	0	3 (60.0%)	1 (20.0%)
N. diffluens (6)	0	1 (16.66%)	1 (16.66%)	4 (66.66%)
N. albida (5)	0	0	3 (60.0%)	2 (40.0%)
T. asahii (1)	1 (100%)	0	0	0
C. jirovecii (1)	1 (100%)	0	0	0
C. orthopsilosis (1)	0	0	0	1(100%)
M. guilliermondii (1)	1 (100%)	0	0	0
C. parapsilosis (1)	3 (60.0%)	0	2(40.0%)	0
R. mucilaginosa (2)	2 (100%)	0	0	0
Total (102)	22 (21.56%)	7 (6.86%)	27 (26.47%)	46 (45.09%)

substantial proteinase activity. Mattei *et al.*[26] discovered that the most efficient proteinase isolates were *C. albicans* and *C. dublinensis*. Oksuz *et al.*[27] investigated the proteinase activities in different *Candida* species; they reported that 52.4% of the examined *Candida* spp. was protease positive.

Phospholipases are enzymes capable of hydrolyzing one or more ester bonds in glycerophospholipids. Due to their connections to epithelial cell adhesion, host cell penetration, epithelial cell invasion, and interaction with the host's signaling pathways, they are considered to be significant virulence factors.[13] Investigation the phospholipase activity of 102 yeast isolates demonstrated that 53 (51.96%) exhibited negative phospholipase activity whereas 4 isolates (3.92%) had weak phospholipase activity, and 10 isolates (9.80%) had moderate phospholipase activity , while 35 isolates (34.31%) exhibited high phospholipase activity (Table 2) The highest percentage of phospholipase activity was observed for *C. albicans* 16(64%). Mothibe and Patel[28] confirmed these findings and Al-laaeiby *et al*.[29] who recorded that *C. albicans* isolates displayed high phospholipase production. Oksuz *et al.* [27] found that 40.9% of the tested isolates had phospholipase activity. They added that the majority of the *C. albicans* isolates examined (53.8%) produced phospholipase. The level of phospholipase production varies with the specific isolate and the site of infection. Samaranayake *et al.*[18] observed that 73% of the examined *C. albicans* isolates displayed phospholipase activity. Ibrahim *et al.*[30] discovered that bloodstream-derived *C. albicans* isolates had higher phospholipase activity than those isolated from the flora of healthy persons. Ramos *et al.*[31] observed that various species of *Candida* recovered from cutaneous lesions were capable to produce proteases and/or phospholipases, these enzymes are multifunctional molecules involved in the infectious process of these yeasts.

The hemolysin activity (Hz) of 102 yeast isolates showed that 55(53.92%) isolates had strong hemolytic activity, whereas 13 isolates (12.74%) exhibited moderate activity, and 11 isolates (10.78%) displayed weak hemolytic activity, and 23 isolates (22.54%) showed negative hemolytic activity. *C. albicans*, *C. glabrata*, *C. dubliniensis*, and *C. krusei* exhibited the highest hemolytic activity in percentages of 96.0, 78.6, 73.3, and 58.8%, respectively (Table 3). Hemolysin is an enzyme that enhances the ability of the pathogens to absorb iron from host tissues, which the fungus uses after infecting the host for metabolism, growth, and invasion.[32] Hemoglobin degraded by hemolysins and became easier to collect the iron element from erythrocytes of the host.[33] The obtained findings were supported by previous studies Luo *et al.*[18], Alrubayae *et al.*[5] *C. albicans* and *C. kefyr* strains exhibited significant hemolytic activity, whereas the other *candida* spp. had lower hemolytic activity .[34] Hemolysins were generated by all *C. albicans* strains that were investigated, whereas 86% of the non-*albicans Candida* spp. displayed hemolytic activity.[10]

The study of biofilm formation by 102 yeast isolates demonstrated that 73 isolates (71.56%) showed biofilm formation ability with an absorbance value more than 0.24. In contrast, 29 isolates (28.43%) revealed inability to form biofilm with an absorbance value less than 0.12. *C. dubliniensis, C. glabrata*, and *C. albicans*, exhibited the highest capacity for biofilm production with percentages of 86.66, 85.71, and 80.00%, respectively (Table 4). According to the intensity of the color, the biofilm producing isolates were qualitatively identified as 25 (24.50%) strong, 17 (16.66%) moderate, and 31 (30.39%) weak. These findings were coincided with Mujumdar *et al.*[35], who found that most *C.* isolates exhibited biofilm formation, and *C. albicans* isolated were the highest for biofilm production. These findings were consistent with a previous study De Freitas *et al.*[36] Adhesion and biofilm development may be impacted by environmental factors, phenotypic switching, and quorum sensing molecules (de Souza *et al.*[37] Recent studies have demonstrated that *C. tropicalis* isolates that produce biofilms have been associated with higher mortality rates in candidemia patients.[38,39] One of the primary yeast

Table 2: Phospholipase activity of pathogen yeast isolates.

Yeast isolates(NO)	*Negative*	*Positive*		
		Weak	*Moderate*	*Strong*
C. albicans (25)	9 (36.0%)	1 (4.0%)	2 (8.0%)	13 (52.0%)
C. krusei (17)	10 (58.82 %)	0	4 (23.52 %)	3 (17.64%)
C. dubliniensis (15)	6 (40.0%)	1 (6.66%)	2 (13.33%)	6 (40.0%)
C. glabrata (14)	11 (78.57%)	0	0	3(21.42%)
C. kefyr (5)	3 (60.0%)	1 (20.0%)	1 (20.0%)	0
C. orthopsilosis (1)	0	0	0	1 (100%)
C. parapsilosis (5)	5 (100%)	0	0	0
C. tropicalis (4)	3 (75.0%)	0	0	1 (25.0%)
C. jirovecii (1)	0	0	0	1 (100%)
M. guilliermondii (1)	1 (100%)	0	0	0
N. albida (5)	2 (40.0%)	0	0	3 (60.0%)
N. diffluens (6)	1 (16.66%)	1 (16.66%)	1 (16.66%)	3 (50.0%)
R. mucilaginosa (2)	2 (100%)	0	0	0
T. asahii (1)	0	0	0	1 (100%)
Total (102)	53 (51.96%)	4 (3.92%)	10 (9.80%)	35 (34.31%)

Table 3: Hemolysin activity of yeast isolates.

Yeast isolates (NO)	*Negative*	*Positive*		
		Weak	*Moderate*	*Strong*
C. albicans (25)	1 (4.0%)	2 (8.0%)	0	22 (88.0%)
C. krusei (17)	7 (41.17%)	2 (11.76%)	5 (29.41%)	3 (17.64)
C. dubliniensis (15)	4 (26.66%)	2(13.33%)	3(20.0%)	6 (40.0%)
C. glabrata (14)	3 (21.42%)	2 (14.28%)	2 (14.28%)	7 (50.0%)
C. kefyr (5)	1 (20.0%)	1 (20.0%)	1 (20.0%)	2 (40.0%)
C. orthopsilosis (1)	0	0	0	1 (100%)
C. parapsilosis (5)	3 (60.0%)	0	1 (20.0%)	1 (20.0%)
C. tropicalis (4)	1 (25.0%)	0	0	3 (75.0%)
C. jirovecii (1)	0	1 (100%)	0	0
M. guilliermondii (1)	0	0	0	1 (100%)
N. albida (5)	1 (20.0%)	0	1 (20.0%)	3 (60.0%)
N. diffluens (6)	0	0	0	6 (100%)
R. mucilaginosa (2)	2 (100%)	0	0	0
T. asahii (1)	0	1 (100%)	0	0
Total (102)	23 (22.54%)	11 (10.78%)	13 (12.74%)	55 (53.92%)

Table 4: Biofilm formation assay of yeast isolates according to absorbance value of biofilm solution stained with crystal violet.

Yeast isolates (NO.)	*Negative*	*Positive*		
		Weak	*Moderate*	*Strong*
C. albicans (25)	5 (20%)	12 (48%)	3 (12%)	5 (20.0%)
C. dubliniensis (15)	2 (13.33%)	6 (40.0%)	3 (20.0%)	4 (26.66%)
C. krusei (17)	10 (58.42%)	2 (11.76%)	4 (23.52%)	1 (5.88%)
C. glabrata (14)	2 (14.28%)	4 (28.57%)	2 (14.28%)	6 (42.85%)
C. kefyr (5)	1 (20.0%)	2 (40.0%)	0	2 (40.0%)
C. orthopsilosis (1)	0	0	0	1(100%)
C. parapsilosis (5)	4 (80.0%)	1 (20.0%)	0	0
C. tropicalis (4)	1 (25.0%)	0	1 (25.0%)	2 (50.0%)
C. jirovecii (1)	1 (100%)	0	0	0
M. guilliermondii (1)	0	0	1 (100%)	0
N. albida (5)	0	1 (20.0%)	2 (40.0%)	2 (40.0%)
N. diffluence (6)	0	3 (50.0%)	1 (16.66%)	2 (33.33%)
R. mucilaginosa (2)	2 (100%)	0	0	0
T. asahii (1)	1 (100%)	0	0	0
Total (102)	29 (28.43%)	31 (30.39%)	17 (16.66%)	25 (24.50%)

virulence factors is biofilm development.[40] These biofilms can establish and remain in a variety of circumstances with an extensive range of nutrients, pH levels, and osmolarities.[41] Polysaccharides have a significant part of the extracellular matrix of *Candida* biofilm, affecting antimicrobial medications' permeability.[42,43] Increased drug tolerance can be brought through the complexity of the structural elements of biofilms, the presence of extracellular matrix, and the activation of efflux pump genes caused by biofilms.[44]

CONCLUSION

The current study focused on the ability of clinical and environmental yeast isolates in the hospital to produce hydrolytic enzymes and biofilms so that all these factors contribute to the establishment and enhancement of the pathogenicity of yeast, as well as the increase of fungal resistance to antifungals by various mechanisms, including preventing drugs from reaching fungal cells through biofilms.

ACKNOWLEDGMENT

The authors gratefully acknowledge the faculty and technical staffs of the microbiology Laboratory, Department of biology, College of Science, University of Basrah, Basrah, Iraq for support.

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