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ORIGINAL ARTICLE

Microbial Analysis of Virulence Factors in Pathogenic Yeasts Isolated from Hospitalized Patients in Najaf Province

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ABSTRACT
Pathogenic yeasts are unicellular eukaryotic microorganisms some of them can cause mild to serious infections in both humans and animals. This study aims to detect some important virulence factor among highly pathogenic yeast strains from different clinical samples. A total of the 160 clinical specimens were collected, which included vaginal, mouth, and diabetic foot swaps, also sputum, stool, and urine specimen. All the clinical samples were diagnosed and identified by morphological and biochemical methods. Some virulence factors were estimated which includes phospholipase, lipase, and proteinase production, also germ tube production, biofilm formation and adhesion ability to the human epithelial cells. The results of swabs cultures showed that 94/60(58.75%) samples were positive for yeast growth. The results of virulence factors assays were as follows: The germ tube and the adhesion test revealed that only C. albicans and C. dubliniensis isolates gave positive results, The biofilm formation test showed that C. albicans isolates were higher in the ability of biofilm formation than the other species. The results of lipase production assay indicated thatC. albicans, C. tropicalis and C. parapsilosis gave positive results, while the phospholipase production assay results were positive for C. albicans, C. dubliensis, C. tropicales, C. parapsilosis , and negative for C. glabrata. The results of Protease production assay indicated that C.albicans ,C.glabrata, C. dubliensi, and C. parapsilosis, were positive for protease production, C.tropicalis was negative. This study concluded that the most predominant species of virulence factors production wasC. Albicans among the clinical isolates, and the most virulence factor produced by the yeast isolates was phospholipase.
Keyword:*Pathogenicyeasts,virulencefactors,Phospholipase,Lipase,Proteinase*

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INTRODUCTION

Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom, whether ascomycetes or basidiomycetes. Most yeasts reproduce asexually by mitosis, and many do so by the asymmetric division process known as budding [1]. In last 30 years there has been a significant increase in the incidence of fungal infections in humans. Such infections may either be superficial, affecting the skin, hair, nails and mucosal membranes, or systemic, involving major body organs. The members of the genus Candida are the most frequently recovered from human fungal infection, Candida genus contains over 150 heterogeneous species [2].*Candida albicans* represents the most commonly implicated species, however, other non albicans species such as *Candida tropicalis, Candida parapsilosis* and *Candida glabrata,* are increasingly being reported as agents of mycoses. Other yeasts of the genus: *Cryptococcus , Rhodotorula ,Pichia, Trichosporon, Malassezia*, and may exceptionally be the cause of superficial mycosis, as they can be responsible for deep systemic infections in immunocompromised patients[3].Like other pathogens, their survival and growth in the host, as well as subsequent host damage, is thought to be mediated by virulence factors which set them apart from harmless microbes[4]. A majority of the diseases caused by *Candida* spp. are due to some type of virulence factors (adhesion, germ tube and biofilm formation, phospholipase, proteins and lipase production) [5].

MATERIAL AND METHODS

A total of the 160 clinical specimens were collected in the present study, which included the 46 vaginal swaps, 58 mouth swaps 3 diabetic foot swaps, 35 sputum specimens, 6 stool specimen and 12 urine specimen who attended the Euphrates Cancer Hospital, AL-Sadder Medical City, AL-Hakem Hospital, and private clinics in Najaf Province, which included 71(44.37 %) males, 79(49.37 %) females and 10(6.25%) children.

Identification of yeast spp.

Culture Examination: The yeasts grew faster than molds on the Sabourauds' Dextroseagar with (Amoxicillin, Tetracycline and Gentamicin) antibiotics and the petri dishes were incubated at 37C° and 25C° separately. The pathological types were considered to have those values on growth at both temperatures. Colonies can be distinguished after 24-48 hours and differ in color, size and luster [6]Then, a microscopic examination of the yeasts was conducted.

Microscopic Examination: A small portion from colony of the culture was placed on a slide with lactophenol cotton blue. Covering with a cover slip and tested under the light microscope [7][8].

Biochemical tests: which includedHiCrome™ Candida Differential Agar**,**This test was performed by inoculating the plates with isolated colony taken from *Candida* isolates culture grown on SDA for 24 hours, and then incubated at 37C° for 24-48 hours and Tobacco agar plates which were streaked with a small amount of inoculums from the isolated colonies. The culture plates were incubated at 28Cº and observed daily up to 96 hours for colony characteristics, such as surface topography (rough or smooth), formation of hyphal fringes at the periphery and color [9].

Germ tube formation test: The analysis was accomplished by adding small portion of each activated isolate to test tube contain 0.5 ml of serum, then all tubes were incubated for 3h at 37C°. After completing the incubation period, 1-2 drops of yeast inoculum were mixed with 10% KOH and explored under a magnification of 40x and 100x of light microscope to observe the attendance / lack of germ tube[2]

Adherence Assay: A drop of mixture (yeast cells with buccal cavity cells) mounted on a glass slide, air-dried, heat-fixed and stained with crystal violet for 1 min and adherence assayed microscopically at 40× lens[10][11].

Biofilm formation Assay with Congo Red Agar Method: The study was carried out by growing the yeast on Congo Red Agar (CRA) after it had been incubated aerobically for 24 to 48 hours at 37C°. The appearance of black dry to dark red colonies suggested a positive result for strong biofilm formation. While isolates Weak biofilm producers remained pink most of the time, biofilm negative strains generated colonies that were white or extremely light pink in color. [12]

Phospholipase Production Assay: Extracellular phospholipase activity assay was done by growing yeast cell on egg yolk agar and measuring the size of the zone of precipitation.[13].

Protease Production Assay The medium contained dextrose 2%, potassium dihydrogen phosphate 0.1%, magnesium phosphate 0.05% and agar 2%. A clear halo zone of clearance around the colony indicated protease production [14].

Lipase Production Assay This test was performed by inoculation Rhan media which prepared previously with isolated coloy taken from yeast species culture grown on SDA for 24hours, and then incubated at 30 C°for 1-5 days. The appearance of white sediment around the grown colonies refers to the positive result [14].

Results and discussion

From the total 160 clinical samples included in the present study 94 (58.75%) samples were positive for yeast growth according to the colony morphology appeared on the fungal selective cultural media supplemented with antibacterial agents, and to the microscopical examination [15].

The results of germ tube revealed that only *C. albicans* and *C. dubliniensis* isolates produced obvious germ tube after incubated them in human serum at 37Cº for three hours, while the isolates of other species did not produce any germ tube. These results were agreed with [25]. The results of adhesion were all the isolates of *C. albicans* and *C. dubliniensis* showed a positive result to adhesion test after incubation with buccal cavity cells for 1h at 37Cº, while the non-albicans *Candida* species isolates showed a negative result. These results were agreed with [17][18]. The biofilm test results showed that *C. Albicans* isolates were higher in the ability of biofilm formation than the other species, from 42 *C. albicans* isolates, there were 36 (85.71%) strong biofilm producers, 6 (14.28%) were considered as weak biofilm producers. This was followed by *C. dubliensis,* from 14 isolates tested 12 (85.71%) were strong biofilm producers, 2 (14.28%) was considered as weak biofilm producers. For *C. tropicales,* from 12 isolates tested 6 (50%) were strong and 6 (50%) were weak biofilm producers. While from 6 *C. parapsilosis* isolates tested, 3 (42.85%) were strong biofilm producers and 1 (14.28%) was weak biofilm producers and 2 (33.33%) isolates did not produce biofilm. One (16.66%) from the 6 *C. glabrata* isolates was formed, strong biofilm producers, 4 (66.66%) were weak biofilm producers and 1 (16.66%) isolate was non-biofilm producers. this agreed with [17].(Table 1).

Table1: Ability of Yeast spp. isolates to biofilm formation.

Yeast spp.	No. of Isolates	Strong biofilm	Weak biofilm	No Biofilm
C. albicans	42	36(85.71%)	6(14.28%)	0(0%)
C. dubliensis	14	12(85.71%)	2(14.28%)	0(0%)
C. tropicales	12	6(50%)	6(50%)	0(0%)
C. parapsilosis	6	3(50%)	1(16.66%)	2(33.33%)
C. glabrata	6	1(16.66%)	4(66.66%)	1(16.66%)
P.kudriavzevii	7	0(0%)	0(0%)	7(100%)
Cr. neoformans	4	0(0%)	0(0%)	4(100%)
R.roseus	2	0(0%)	0(0%)	2(100%)
M. capitatus	1	0(0%)	0(0%)	1(100%)
Total	94	58	19	17

C.albicans remains the species that most associated with very high biofilm formation, this can be explained by have the genetic ability to adhere and form a thick extracellular matrix and produces highly structured biofilms composed of multiple cell types encased in an extracellular matrix. [19][20].

The results of lipase production assay indicated that *Candida* species were differed in their ability to produce lipase.*C. albicans*, *C. tropicalis* and *C. parapsilosis* gave positive results by producing white sediments around the grown colonies after incubated their colonies on Rhan medium for 72 h at 30ºC (Figure 1).These results were agreed with [21].Lipase has very important role in *Candida* species pathogenicity because it is able to digest lipids for nutrient acquisition, adhesion to host cells and host tissues [15](Table 2).

Table 2: Lipase,Phospholipase, andProteinase production byYeast spp. isolates

Candia spp.	Isolates No.	Lipase	Phospholipase	Proteinase
C. albicans	42	20(47.61%)	25(59.52%)	17(40.47%)
C. dubliensis	14	2(14.28%)	7(50%)	6(42.85%)
C. tropicales	12	3(25%)	8(66.66%)	0(0%)
C. parapsilosis	6	1(16.66%)	4(66.66%)	3(50%)
C. glabrata	6	4(66.66%)	0 (0 %)	5(83.33%)
P.kudriavzevii	7	0(0%)	0(0%)	0(0%)
Cr. neoformans	4	0(0%)	0(0%)	0(0%)
R.roseus	2	0(0%)	0(0%)	0(0%)
M. capitatus	1	0(0%)	0(0%)	0(0%)

Figure 1: Lipase production test: *C. albicans, C. tropicalis* and *C. parapsilosis* gave positive results by formation white sediments around the grown colonies on Rhan medium.

The phospholipase production assay indicated that *Candida* species were differed in their ability to produce this enzyme.The results were positive for *C. albicans, C. dubliensis, C. tropicales,C. parapsilosis* , and negative for *C. glabrata* to its production (Table 2),(Figure2),These results are similar to that found by [22].The extracellular phospholipases of *Candida* species have a significant role in the pathogenesis of infections and invasion to mucosal epithelia [23].

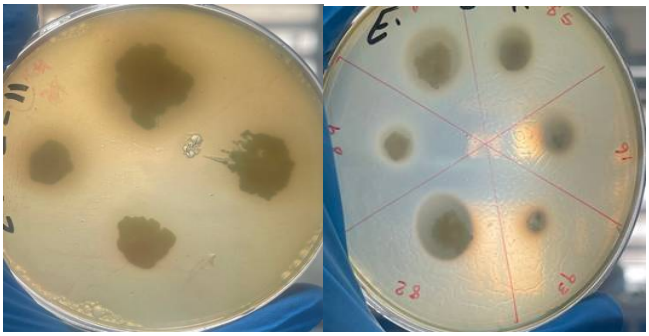

Figure 2: Phospholipase production test: *C. albicans,C. dubliensis, C. parapsilosis,C.tropicalis* isolates gave positive results by formation halo zone around the grown colonies on egg yolk agar medium, *C. glabrata*, isolates gave negative results.

The results of Protease production assay indicated that *Candida* species were differed in their ability to produce Protease enzyme. *C.albicans* ,*C.glabrata, C. dubliensi,* and *C. parapsilosis,* were positive for protease production, *C.tropicalis* was negative. This result agreed with [24] (Table 2), (Figure 3).protease activity was considered to play important roles in the pathogenesis of opportunistic fungi. The roles of this hydrolytic enzyme in *C. albicans* and other yeast species seem to be related to its virulence [14].

Figure 3: Protease production test: *C.albicans , C.glabrata ,C. dubliensis,* and *C. parapsilosis* isolates gave positive results by formation halo zone around the grown colonies on Rhan agar medium, *C.tropicals* isolates gave negative results.

CONCLUSION

The most predominant species of virulence factors production was *C. albicans* among the clinical isolates, and the most virulence factor produced by the yeast isolates was phospholipase.

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