Iran. J. Ichthyol. (Special Issue 2022): 61-68

P-ISSN: 2383-1561; E-ISSN: 2383-0964
http://www.ijichthyol.org

Research Article

Evaluation of virulence factors of clinical yeast isolates from nosocomial fungal infections with the determination of their antifungal susceptibility profile

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Abstract
Due to an increased resistance to antifungal medicines, the prevalence of hospital-acquired fungal infection has recently increased. This study aimed to determine the virulence features that assist in increasing the pathogenicity of clinical yeast isolates. For this purpose, 61 clinical yeast isolates were used to examine their enzymatic activity (proteinase, phospholipase, and hemolysin), potential to form biofilms, and antifungal susceptibility patterns. The results indicated that majority of the yeast isolates exhibited potential proteinase activity, with 24.6% exhibiting weak activity, 19.7% moderate activity, and 9.8% high activity, while 45.9% phospholipase activity, with 16.4% weak activity, 24.6% exhibiting moderate activity, and 4.9% strong activity. While hemolysin production was demonstrated in 85.2%, that 59.0% were strong, 21.3% moderate, and 4.9% weak. Additionally, it was possible to identify biofilm development, which occurred in 90.2% of isolates. All isolates showed sensitivity to the antifungals tested, with the exception of one *Candida glabrata* isolate that demonstrated resistance to voriconazol and two *Candida parapsilosis* isolates resistance to flucytosine. The results also revealed no significant changes in proteinase activity or drug susceptibility profile, but significant variations in phospholipase, hemolysin, and biofilm generation amongst yeast isolates.

Keywords: Nosocomial infections, Candida, Enzymatic activity, Biofilm, Antifungal susceptibility test.

Citation: Abeed, F.K. & Alrubayae, I.M.N. 2022. Evaluation of virulence factors of clinical yeast isolates from nosocomial fungal infections with the determination of their antifungal susceptibility profile. Iranian Journal of Ichthyology 9(ICAB, Special issue 2022): 61-68.

Introduction

In the last decades, the frequency of hospital-acquired fungal infections has risen (Joshua et al. 2006). New technologies and therapies, such as bone marrow or solid-organ transplants and chemotherapeutic agents have become common at many medical centers, resulting in many immunocompromised individuals. Invasive monitoring equipment, parenteral nourishment, broad-spectrum antibacterial medicines, and assisted ventilation, as well as treatment in specialist units, have helped to treat patients with previously lethal or deadly conditions and have given life to preterm infants previously considered to be nonviable. However, these achievements have resulted in an increase in the number of extremely sick, immunocompromised, hospitalized patients. In addition, the AIDS pandemic has added to the rising number of immunocompromised people. These immunocompromised patients are highly susceptible to nosocomial infections caused by organisms such as fungi that were previously considered to be of low virulence or “nonpathogenic”. Fungal infections in these patients are often severe, rapidly progressive, and difficult to diagnose or treat. Fungi are eukaryotic cells; they are more complex than bacteria. A thorough appreciation and understanding of fungal infections, including diagnostic and

therapeutic modalities, are needed among clinicians and microbiologists to provide better patient care (Fridkin & Jarvis 1996).

Among various fungal pathogens, C*andida* spp. cause substantial morbidity and mortality in hospitalized patients, especially among critically ill hospitalized patients. The frequency of non-albicans spp. of *Candida (*NAC) causing nosocomial infections is increasing. Candida infections are usually endogenous; however, exogenous infections may also occur. The important predisposing factors leading to nosocomial candidiasis include treatment with broad-spectrum antibiotics, immunosuppression, malignancy, surgical intervention, diabetes and prolonged hospitalization. Virulence factors like adherence to biotic and abiotic substances and the production of hydrolytic enzymes play an important role. Also, the biofilm-forming ability makes it more noxious. Nosocomial Candida infections are difficult to diagnose clinically and refractory to therapy. Therefore, rapid and accurate laboratory diagnosis is important to provide appropriate antifungal treatment (Jahagirdar et al. 2018). Hence, this study aimed to identify the virulence features viz. enzymatic activity, potential to form biofilms, and antifungal susceptibility patterns that assist in increasing the pathogenicity of clinical yeast isolates.

Materials and methods

Yeast isolates: A total of 61 clinical yeast isolates from different sources of nosocomial infections were obtained from the laboratory of clinical and physiological researches, Department of Biology, College of science, University of Basrah, Basrah, Iraq. They include *Candida albicans* (28)*, C. glabrata* (9)*, C. tropicalis* (8)*, C. parapsilosis* (6)*, C. krusei* (3)*, C. dubliniensis* (3)*, C. lusitaniae* (2)*, Naganishia globosa* (1), and *N. diffluens* (1) and were initially identified with methods of colony colours on Brilliance Candida agar, formation of germ tube after three hours incubation in human serum, and chlamydospore development and carbohydrate utilization analysis.

Enzymatic activities

Elaboration of yeast inoculum: The suspension of activated yeast isolates was prepared by adding small portions of yeast colony to 5ml 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 was measured using bovine serum albumin (BSA) based on Aoki et al. (1990). 10µl 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. 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 (Price et al. 1982) 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; Prz >0.5, strong positive; Prz >0.5, strong positive.

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. (1984). The egg yolk agar medium was inoculated with 5µl of 1×10^8 CFU/ml of yeast suspension in addition to 5µl of sterile normal saline as a control plate. The diameter of the precipitation zone around the colony was indicated phospholipase activity which was measured after two days' incubation period at 37°C. Phospholipase activity (Pz value) was calculated as the ratio of colony diameter to total colony diameter plus precipitation zones and graded as described above.

Hemolysis activity: Seven ml of fresh sheep blood was added to 100 ml SDA enriched with glucose at a final concentration of 3% (w/v) to make the medium that was used to evaluate yeast isolates of hemolysin

production (Luo et al. 2001). The medium was inoculated with a standard inoculum of yeast isolates (10µl of 1×10^8 CFU/ml), while the control plate was inoculated by 10µl of sterile normal saline. After that, the plates were incubated for 48 hours at 37°C in 5% CO_2. The appearance of a clear transparent zone surrounding the inoculum site after incubation showed good hemolytic activity. Therefore, the hemolytic index (Hz value) was calculated by dividing the colony diameter by the entire diameter of the colony plus the transparent halo according to (Sriphannam et al. 2019). All tests of yeast isolates were carried out duplicate with control plates that inoculated with sterile normal saline.

Biofilm formation test: Two methods were used to detect the biofilm formation of yeast isolates as follow:

Congo red agar method: Freeman et al. (1989) described Congo Red Agar (CRA) as a simple and qualitative technique for detecting biofilm development. CRA plates were inoculation with test isolates that activated on SDA for 2-5 days previously, then, incubated aerobically for 24 hours at 37°C. The appearance of red colonies was indicated to the strong activity of biofilm, while moderate and negative production of biofilm was shown as pink and white colonies respectively.

Microtiter plate technique: Pre-sterilized 96-well polystyrene microplates were used to assess biofilm development (Sida et al. 2016). For each isolate, a suspension was adjusted to 1 McFarland to equal $1*10^8$ CFU/ml. Each well of the microplate was filled with 180ml of Sabouraud dextrose broth supplemented with 8% glucose, followed by adding 20µl of the tested yeast suspension, and then microtiter plates were covered and incubated at 37°C for 24 hours. The wells were emptied from media and washed three times with a sterile phosphate buffer solution (PBS). After that, microtiter plates were stained with 1% Safranin for 5 minutes before being read by an ELISA reader at 630 nm to investigate the percentage transmittance (% T). All tests were carried out in triplicate, and the average was computed. Finally, adherent biofilm layers were classified as negative, weak (+) (% T<20), moderate (++) (% T = 20-35), strong (+++) (%T = 36-50), or very strong (++++) (% T>50).

Antifungal susceptibility test: The susceptibility assay of fluconazole, voriconazole, amphotericin B, flucytosine, caspofungin, and micofungin was subjected against 61 clinical yeast isolates using Vitek 2 program according to the manufacturer's recommendations.

Results and Discussion

During the last two decades, yeasts of the genus *Candida* have been demonstrated as one of the most important etiological agents of hospital-acquired infections. Mycoses are most commonly caused by pathogenic *Candida* species, especially *C. albicans*, *C. tropicalis*, and *C. parapsilosis* that recognized as increasingly important nosocomial pathogens (Vijayalakshmi et al. 2016). *Candida* has been suggested diverse virulence properties that can aid the transition of a mucosal colonizer to a lethal disseminating pathogen. However, potential enzymes like proteinases, phospholipases, and lipases have an important role to enhance the penetration through cells, while adhesions help the yeast in adhering to the host cell surfaces. At the same time, host defense shows beneficial function to restrict fungal infections to each local or invasive configuration that appeared in immunocompetent and immunocompromised hosts respectively (Tellapragada et al. 2014). Secreted aspartyl proteinases (SAP) in the genus *Candida* are familiar to raise the hyphal formation, epithelial cell injury, invasion, and inflammatory responses. *In vivo* experimental models also demonstrated an increase in the invasiveness of yeasts with the production of proteinases (Tellapragada et al. 2014).

After 7 days of incubation at 37°C, the proteinase activity (Prz) of 61 yeast isolates was evaluated by forming a halo zone surrounding the inoculation region on BSA medium. 28 yeast isolates (45.9%) were determined as negative members of proteinase

Table 1. Proteinase activity of pathogenic yeast isolates isolated from hospital acquired fungal infections.

Yeast isolates	Negative	Positive		
		Weak	Moderate	Strong
Candida albicans (28)	8 (28.6%)	7 (25.0%)	9 (32.1%)	4 (14.3%)
C. glabrata (9)	7 (77.7%)	2 (22.3%)	0	0
C. tropicalis (8)	4 (50.0%)	3 (37.5%)	1 (12.5%)	0
C. parapsilosis (6)	2 (33.3%)	2 (33.3%)	1 (17%)	1 (17%)
C. krusei (3)	2 (66.6%)	1 (33.4%)	0	0
C. dubliniensis (3)	2 (66.6%)	0	0	1 (33.4%)
C. lusitaniea (2)	2 (100%)	0	0	0
Naganishia globose (1)	0	0	1(100)	0
N. diffluens (1)	1(100)	0	0	0

Table 2. Phospholipase activity of nosocomial fungal etiological pathogens.

Yeast isolates	Negative	Positive		
		Weak	Moderate	Strong
Candida albicans	5(17.9%)	7 (25.0%)	13(46.4%)	3(10.7%)
C. glabrata	9 (100%)	0	0	0
C. tropicalis	7(87.5%)	1 (12.5%)	0	0
C. parapsilosis	6 (100%)	0	0	0
C. krusei	2 (66.6%)	1 (33.4%)	0	0
C. dubliniensis	2 (66.6%)	0	1 (33.4%)	0
C. lusitaniea	2 (100%)	0	0	0
Naganishia globosa	0	0	1(100)	0
N. diffluens	0	1(100)	0	0

High significant differences ($P \leq 0.01$)

production assay, while 15 (24.6%) of them were showed weak activity, in addition to 12 (19.7 %) and 6 (9.8%) isolates were appeared moderate and high activity, respectively without any significant differences ($P>0.01$) of yeast isolates (Table 1). The result of the present finding was disagreed with previous studies (Othman et al. 2018; deMelo et al. 2019; Al-Rubayae et al. 2020) that showed the majority of tested isolates with have proteinase activity. This may be due to the source of samples or the isolates were subjected to stress that affected their ability to produce proteinase. Furthermore, *C. albicans* was showed the highest ability to produce proteinase (71.4%). *Candida albicans* is the leading producer of proteinases among the species of *Candida*, and recent studies suggest the production of 4 types of SAP by *C. tropicalis* too (Tellapragada et al. 2014; Al-Rubayae et al. 2020; Al-laaeiby et al. 2020).

Phospholipases are hydrolytic enzymes that offensive phospholipids in cell membranes. They play an active role in the invasion of host tissue by disrupting the epithelial cell membranes and allowing the hyphal tip to enter the cytoplasm, leading to cell lysis and tissue damage (Sriphannam et al. 2019). The current study revealed that the majority of isolates (33) appeared negative phospholipase activity (54.1%); 10 isolates (16.4%) had weak phospholipase activity, and 15 isolates (24.6%) had moderate phospholipase activity, while

Table 3. Hemolysin activity of pathogenic yeast isolates.

Yeast isolates	Negative	Positive		
		Weak	Moderate	Strong
Candida albicans	2 (7.1%)	2 (7.1)	6 (21.4%)	18 (64.4%)
C. glabrata	4 (44.4%)	0	0	5 (55.6%)
C. tropicalis	1 (12.5%)	0	0	7 (87.5%)
C. parapsilosis	1 (16.7%)	0	2 (33.3%)	3 (50%)
C. krusei	1 (33.3%)	0	2 (66.7%)	0
C. dubliniensis	0	0	2 (66.7%)	1 (33.3%)
C. lusitaniea	0	0	0	2 (100%)
Naganishia globosa	0	0	1(100)	0
N. diffluens	0	1(100)	0	0

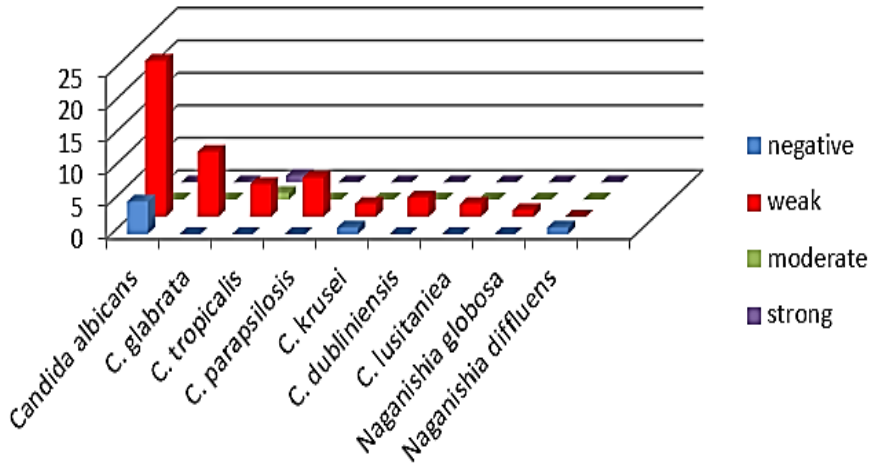

Fig.1. Biofilm formation of yeast isolates according to absorbance value of biofilm solution stained with safranin.

three isolates (4.9%) high phospholipase activity (Table 2). The yeast isolates of *C. glabrata, C. parapsilosis*, and *C. lusitaniea* were detected their inability of phospholipase production, so that, our result was conflicted with finding of de Melo et al. (2019), while *C. albicans* was showed high potential to produce phospholipase, therefore this is in agreement with studies of Alrubayae et al. (2013), Al-laaeiby et al. (2020) and Alrubayae et al. (2020).

Hemolytic capacity is an important virulence factor that allows fungi of the genus *Candida* to acquire iron from host tissues, which then is used by the fungus for metabolism, growth and invasion during host infection which starts *in vivo* by binding the yeast cells with erythrocytes through receptors of the complement system, then, produces a hemolysis element that induces lysis of the erythrocyte. However, the mechanism and molecular basis of hemolysis caused by *C. albicans* remain unknown (Rossoni et al. 2013). After 48 hours at 37°C in 5% CO_2, the hemolysin activity (Hz) of 61 yeast isolates with a transparent halo surrounding the inoculation region were evaluated on SDA supplemented with 7% sheep blood and 3% glucose. 36 isolates (59.0%) had strong hemolytic activity, 13 isolates (21.3%) had moderate activity, while 3 isolates (4.9%) and 9 isolates (14.8%) had weak and negative hemolytic activity, respectively (Table 3). In this test, it was found that most of the isolates had the ability to secrete hemolysin enzyme by 83%, highly significant differences, this matched with previous studies (Othman et al. 2018; Al-Rubayae et al. 2020; El-

Table 4. Biofilm formation assay of clinical yeast isolates according Congo red agar method.

Yeast isolates (No)	Negative (%)	Positive (%)
Candida albicans(28)	4 (17.3)	24 (82.7)
C. glabrata(9)	0	9 (100)
C. tropicalis(8)	0	8 (100)
C. parapsilosis (6)	0	6 (100)
C. krusei (3)	1 (33.4)	2 (66.6)
C. dubliniensis (3)	0	3 (100)
C. lusitaniea (2)	0	2(100)
Naganishia globose (1)	0	1(100)
N. diffluens (1)	1(100)	

Table 5. Activity of antifungal against to yeast isolates.

Yeast isolate No	Antifungal											
	*Fluconazole		*Voriconazole		*Amphotericin B		*Flucytosine		*Caspofungin		*Micafungin	
	*S	*R	S	R	S	R	S	R	S	R	S	R
Candida albicans (28)	28	0	28	0	28	0	28	0	28	0	28	0
C. glabrata (9)	9	0	8	1	9	0	9	0	9	0	9	0
C. tropicalis (8)	8	0	8	0	8	0	8	0	8	0	8	0
C. parapsilosis (6)	6	0	6	0	6	0	4	2	6	0	6	0
C. krusei (3)	3	0	3	0	3	0	3	0	3	0	3	0
C. dubliniensis (3)	3	0	3	0	3	0	3	0	3	0	3	0
C. lusitainiea (2)	2	0	2	0	2	0	2	0	2	0	2	0
Naganishia globosa (1)	1	0	1	0	1	0	1	0	1	0	1	0
Naganishia diffluens(1)	1	0	1	0	1	0	1	0	1	0	1	0

Kholy et al. 2021).

Biofilm production is considered as one of the most potent pathogenic traits attributed to treatment failures and recurrent infections (Tellapragada et al. 2014). Tallaprageda et al. (2014) recognized a high rate of biofilm activity among the *Candida* isolates from blood and other invasive infections. While Hassan et al. (2009) found that an outstandingly higher number of *C. albicans* isolates were biofilm producers in compassion with non-albicans *Candida*. Though, the exact reason for the higher rate of biofilm production in *C. albicans* is unclear, Further studies like scanning electron microscopy of complex biofilm architectures assist the solidity and intensity of these biofilms to the huger number of hyphal parts produced by *C. albicans* than *C. tropicalis* and *C. parapsilosis* (Hassan et al. 2009). The biofilm formation assay was performed by two styles (Congo red agar and polystyrene microtiter plates) (Fig. 1, Table 4). The quantification Safranin technique on polystyrene microtiter plates was used to demonstrate biofilm development in yeast isolates. Out of the 61 yeast isolates examined, 55 (90.2%) had the potential to produce biofilms. Six isolates (9.8%) were unable to form biofilms with an absorbance value of zero. This result is in agreement with previous studies finding of *C. albicans* ability biofilm formation (Al-Rubayae et al. 2013; de Melo et al. 2019; Al-laaeiby et al. 2020; Al-Rubayae et al. 2020) as sequentially 66, 97, 95 and 100%, respectively)

Resistance to antifungal agents has increased during the last decade. Therefore, identification of *Candida* spp. and its antifungal drug susceptibility are vital in the management of *Candida* infections (Sriphannam et al. 2019). Table 5 shows that all antifungal agents inhibit yeast isolates except *C. glabrata* that showed resistance to voriconazol in addition to two isolates of *C. parapsilosis* that showed opposition to flucytosine, as well as, the

statistical analysis was not detected any significant difference among antifungals and different isolates. Our result of the antifungal susceptibility assay is in agreement with Alrubayae et al. (2020) and disagreement with Karwan et al. (2018) that showed that all *Candida* spp. which isolated from urine resistance to Fluconazole.

Conclusions

The current study focused on the ability of clinical yeast isolates from hospital-acquired fungal infections to produce hydrolytic enzymes, biofilm, and determine drug susceptibility patterns so that all of these factors contribute to the establishment and enhancement of yeast pathogenicity, as well as increased resistance to antifungal treatment by various mechanisms, including preventing drugs from reaching fungal cells through biofilm formation.

Acknowledgement

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

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