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Original Research Paper:

Relationship of biofilm strength levels in *Candida albicans* isolates with the type of clinical specimens

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Abstract: The virulence factors in C. albicans such as biofilm formation, germ tube, adhesion, hydrolytic enzymes, such as lipases, proteases, phospholipases, and hemolysin and catalase enzyme that allow successful colonization and infection of the host under suitable predisposing conditions. A total of 151 clinical specimens were collected from immunocompromised patients suffering from underlying diseases included cancer, diabetes, and thalassemia. All the collected specimens were cultured on yeast selective media. From the total clinical specimens 111 (73.50%) specimens were positive for yeast growth, among them the most predominant species was C. albicans at 64 (57.65%). There was a difference in the prevalence of yeast species isolates according to the patient's gender, which it was higher in females 76 (68.46%) than in males 35 (31.53%), The virulence factors results showed that all isolates belonging to the species C.albicans form the germ tube when incubated at a temperature of 37°C for 3 hours in 0.5 ml of human serum while all the isolates of C. albicans showed a positive result to adhesion test after incubation for 1h at 37C° The test results showed that from 64 C. albicans isolates, there were 16 (25%) strong biofilm producers, 6 (14.28%) were considered as weak biofilm producers. The studies described here form the basis for investigations into the mechanisms of Candida biofilm formation and provide the means to design novel therapies for biofilm-based infections.

Keywords: candida albicans, The virulence factors, biofilm formation, immunocompromised patients.

1.Introduction

Mold infections are considerably fewer common than fungal infections, which are potentially fatal opportunistic infections that have become a major source of disease and death in critically sick patients [1,2].

The primary cause of fungal infections is *Candida* spp. Members of the natural microbiota of the mucosal oral cavity, gastrointestinal system, and vagina, *Candida* spp. cause a range of clinical symptoms, from bloodstream infections to mucocutaneous overgrowth [3].

One of the fungus that poses the greatest threat to human health is *Candida albicans*. Even though this yeast is a typical part of the commensal flora, it can infect both healthy and immunocompromised individuals' skin, mouth, vagina, and gut. In addition, hospitalized patients who develop invasive candidiasis—an infection of the heart, blood, and other organs—are the victims of *C. albicans*. 50% of individuals who have invasive candidiasis die from it, even if they are otherwise healthy [4,5].

Under the right predisposing conditions, virulence factors in *Candida albicans* include biofilm formation,

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adhesion, germ tube production, and hydrolytic enzymes such lipases, proteases, phospholipases, hemolysin, and catalase that facilitate effective colonization and infection of the host. Additionally, the colonizing oral population may act as a reservoir for diseases that have the potential to be fatal. Understanding these virulence factors will be crucial for comprehending the pathophysiology of candidiasis and for identifying novel targets for antifungal medications to be used in more effective treatment plans [6].

As the most invasive form of yeast, hyphae play a crucial role in the development of the illness since they are required for systemic infections to diffuse into the bloodstream. Furthermore, the development of other virulence factors, including adhesions, invasions, metal acquisition factors, hydrolytic and detoxifying enzymes, frequently coincides with the creation of hyphal colonies. These factors all contribute to the pathogenesis of *Candida albicans* [7,8].

Some of the factors that can lead to multidrug resistance (MDR) include the formation of biofilms, which reduce the accessibility of the antifungal; selection of spontaneous mutations that increase expression or decreased susceptibility of the target; altered chromosome abnormalities; overexpression of multidrug efflux pumps; and the capacity to evade host immune defenses [9].

Recent years have seen an increase in the crucial issue of biofilm development by different microorganisms in both human and veterinary medicine. A microbial community that is organized, bound to a biotic or abiotic surface, and shielded by an extracellular matrix is called a biofilm. One type of bacteria or a mixed culture of bacteria and yeast can create the biofilm [10]. By offering a safe refuge that is resistant to therapy, biofilms can either start or extend infections by allowing cells to infiltrate local tissue and concurrently create new infection sites. They may also cause medical devices that have been implanted to malfunction [11].

Numerous variables and processes involving both the host organism and the yeast affect the production of biofilms. In 38–72 hours, *Candida* spp. can produce a biofilm. The yeast's attachment to a living or non-living surface is the initial step in this intricate process, which has multiple stages. Cell growth and the initial step of adherent cell filamentation make up the following stage of biofilm formation. The development of the biofilm comes next. The dispersion stage, in which some yeast cells break out from the biofilm and enter the surrounding environment, is the last stage of biofilm growth. The type of Candida determines how different the biofilm development process is. The interplay between *Candida* spp. and host homeostasis and variation, such as mucosal pH shifts or dietary changes, as well as the host's immune system status, affects the yeasts' ability to multiply and form biofilms. [11,12].

Adhesion is a crucial virulence factor for *Candida* and other yeast-like fungi that have been identified as having medical significance. It is thought to be the initial stage of infection into the host tissues. Furthermore, the hydrophobic contacts between the surfaces of the epithelial cells and yeast cells allow *Candida* to attach itself to human epithelial cells [13,14].

Despite the fact that *C. albicans* cells generally /reproduce via budding, which results in the creation of yeast cells, they commonly create germ tubes in unfavorable circumstances. When germ tubes are produced, the organism transforms into a mycelial or filamentous development. These morphological changes frequently indicate how the fungus responds to shifting environmental circumstances and may help it fit into various biological niches[15].

2. Methodology

Samples collection

A total of the 151 clinical specimens included 111 (73.5%) specimens from the patients with cancer, 32 (21.1%) specimens from the patients having Diabetes, and 8 (5.2%) from Thalassemia patients who attended the Euphrates Cancer Hospital, AL-Sadder Medical City, and AL-Zahraa Educational Hospital in Najaf Province, which included 45 (29.8%) males and 106 (70.1%) females.

Yeast species identification

We used the previously listed classification keys [16,17,18] in line with the following guidelines to diagnose a few phenotypic (microscopic and cultural) and biochemical traits of yeasts:

A. Culture examination: On Sabouraud's Dextrose agar supplemented with antibiotics (Amoxicillin, Tetracycline, and Gentamicin), the yeasts outgrown the molds, and the petri dishes were incubated at 37°C and 25°C independently. Those data on growth at both temperatures were thought to belong to the abnormal categories. After 24 to 48 hours, colonies can be identified by their differences in size, color, and shine[19]. The yeasts were then examined under a microscope.

B. Microscopic Examination**:** A tiny piece of the culture colony was put on a lactophenol cotton blue slide. Testing under a light microscope and covering with a cover slip [20,21].

HiCrome™ Candida Differential Agar test: In order to perform this test, HiCromeTM Candida Differential Agar was inoculated with an isolated colony from a culture of *Candida* isolates established on SDA for 24

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hours. The colony was then incubated at 37°C for 24 to 48 hours. According to the manufacturer's instructions, the assay was used to presumptively identify *Candida* spp. by color of the resulting growth: *C. albicans* =Light green, *C. dubliensis* = dark green, *C. tropicalis* = Metallic blue with pink halo, *C. parapsilosis* = white to purple, and *C. glabrata* = pink with white margins [22]. However, pale pink colonies are produced by species of the *C. haemulonii* complex, such as *C. auris* and others like *C. krusie* [23].

Virulence factors assay

Germ tube formation test: A yeast test used to distinguish between species belonging to the genus Candida spp. because the two types *C. albicans* and *C. dubliniensis* are characterized by their ability to form this tube that is in the form of a long extension from the cell surface and has a broad base of attachment to the cell, so it can be distinguished from the pseudo-hyphae, which have a constricted attachment at the point of origin. Germ tubes do not exhibit constriction at the point of origin[24]. The analysis was conducted by putting a little part of each activated isolate to a test tube containing 0.5 ml of serum, then all tubes were incubated for 3 hours at 37°C. After completing the incubation period, 1-2 drops of yeast inoculum were combined with 10% KOH and explored under a magnification of 40x and 100x of light microscope to observe the presence / absence of germ tube [19].

Adherence assay: The Candidal adhesion experiment was carried out as follows: *Candida* isolates from an overnight culture on Sabouraud's dextrose agar were harvested using a loop and floated in PBS with a turbidity adjusted to match a 0.5 McFarland density standard, yielding a suspension of 5×106 yeast cells/ml. Then, 1 ml of this solution was combined with 1 ml of a mixture containing buccal cavity cells prepared by swabbing the buccal mucosa and suspending in PBS. The mixture was incubated in a shaking incubator at 80 rpm and 37°Cº for 1 hour. A drop of this combination was put on a glass slide, air-dried, heat-fixed, and stained with crystal violet for 1 minute. Adherence was assessed microscopically at a 40× lens [25,26].

Biofilm formation assay with congo red agar method The yeast was grown on Congo Red Agar (CRA) after being aerobically incubated at 37 degrees Celsius for 24 to 48 hours. The presence of black dry to dark red colonies indicated a favorable outcome for vigorous biofilm growth. While isolates of weak biofilm makers remained pink for the most part, the colonies' cores darkened slightly. Biofilm negative strains produced colonies that were white or very light pink in hue. The experiment was conducted three times in duplicate[27].

3. Results and discussion

Specimens were collected in table (1) from the total 151 clinical specimens included in the present study 111 (73.5%) specimens from the patients with cancer, 32 (21.1%) specimens from the patients having diabetes, and 8 (5.2%) from thalassemia patients while the Figure (1) shows the differences in the distribution of isolates among clinical specimens, the greatest number of yeast isolates were in the urine specimens which were 39 specimens (74.35%) followed by oral swap specimens 112 specimens (72.21%).

Table 1: Prevalence of *Candida* species isolates among patients.

Type of patients	No. of specimens	Percentage
Cancer patients	111	73.5%
Diabetics	32	21.1%
Thalassemia patients	8	5.2%

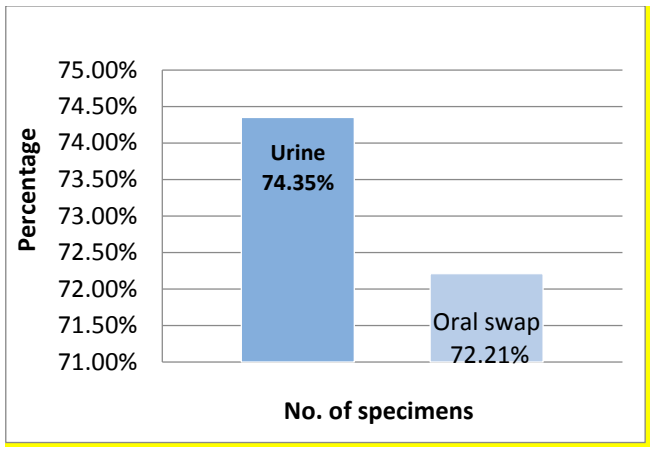

Figure 1: Prevalence of *Candida* species isolates among specimens.

Several factors contribute to the rise in candidiasis in cancer patients, including It is widely acknowledged that cytotoxic chemotherapy, radiation therapy, or cancer itself can decrease cell-mediated host immunity, which is essential for avoiding fungal infections. Chemotherapy and radiation therapy both have the potential to produce mucositis, which is inflammation and irritation to the oral mucosa that can result in xerostomia and hyposalivation. This, in turn, promotes the proliferation, colonisation, and infection of oral yeast. When oral candidiasis causes pain or burning, it can lead to poor nutrition and even more serious illnesses such as candidemia or esophagitis. This can

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lead to a large increase in morbidity. Corticosteroids, broad-spectrum antibiotics, and diabetes are other risk factors associated with the development of this oral disease[28,29,30,31].

Prevalence of pathogenic yeast isolates by gender

The results revealed that the prevalence of yeast species isolates was much greater in females 76 (68.46%) than in males 35 (31.53%).

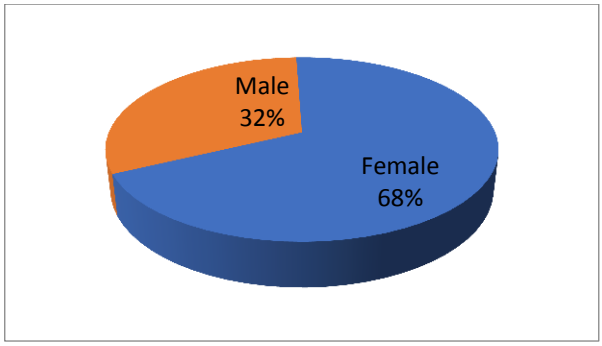

Figure 2: Prevalence of pathogenic yeast isolates according to gender.

The physical and physiological differences between the sexes are the main reason why candidiasis is more common in girls than in males. Among other parts of the body, the skin, mouth, and genital tract all contain small amounts of *Candida* spp. especially in the vaginal environment, where yeast may colonize and thrive more easily due to its increased surface area compared to the male genitalia. This makes the ideal conditions for yeast overgrowth warm and moist especially when hormonal fluctuations take place (during the menstrual cycle, during pregnancy, or during menopause), or when specific drugs such as antibiotics or birth control pills are consumed. Hormone fluctuations, particularly those involving estrogen, can also affect the vaginal ecology. Overeither estrogen may encourage the growth of yeast. Pregnancy, hormone medication, or changes in hormone levels during the menstrual cycle can all increase a woman's chance of developing female candidiasis [32,33].

Cultural characteristics

To identify *C. albicans*, cultural traits served as a foundation. All specimens were cultivated on Sabouraud dextrose agar plates (SDA), which are perfect for the cultivation of pathogenic and opportunistic fungi [34].

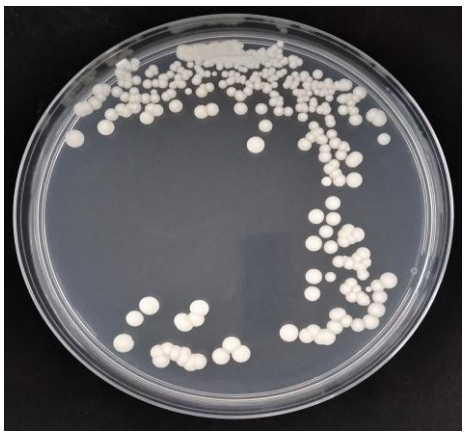

Figure 3: Growth of *Candida Albicans* on SDA at 37°C for 48 hr.

Microscopic characteristics

The low cost and rapid diagnostic turnaround of microscopic inspection are two of its many benefits. These attributes make it perfect for identifying whether a growth is a yeast isolate based on traits like budding development[35].

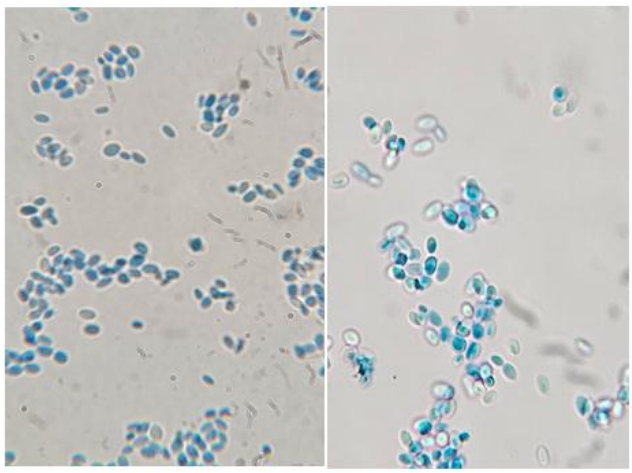

Figure 4: Microscopic feature of yeast spp. isolates pigmented with lactophenol.

Biochemical identification of Candida species using HiCrome™ Candida Differential Agar medium

The investigation of *Candida* spp. on HiChrome™ Candida Differential Agar revealed differences in color after incubation at 37°C for 24 hours[36]. the recovered colonies were identified based on the color of the manufacturer's hichrome. This method allows for quick identification of most *Candida* species and distinguishes them from other clinical *Candida* species. *C. albicans* smooth colonies were light

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green, *C.tropicalis* metallic blue with pink halo, *C.glabrata* pink with white margins, *C.dubliniesis* dark green, and *C. haemulonii* complex such as *C. auris* and others like *C. krusie* showed pink color with white peripheral as shown in the table (2) ; figure (5).

Table 2: *Candida* spp. isolates colony color on HiChrome™ Candida Differential Agar.

Yeasts specie	Color of colony	NO. of isolate
C. albicans	Light green	64 (57.65%)
C. glabrata	Pink with white margins	17 (15.31%)
C. dubliensis	Dark green	9 (8.1%)
C. parapsilosis	White to purple	3 (2.7%)
C. tropicalis	Metallic blue with pink halo	9 (8.1%)
C. haemulonii complex such as *C. auris* and others like *C. krusie*	Pale pink	10 (9%)

Figure 5: Growth of yeast isolates on HiCrome™ Candida Differential Agar shows different colony colors.

Germ tube production test

Although *C. albicans* cells multiply regularly by budding and forming yeast cells, they frequently create germ tubes in poor conditions. The formation of germ tubes leads to filamentous growth or mycelial shape. These morphological transformations frequently represent the fungus' response to changing environmental conditions and may allow the fungus to adapt to diverse biological niches [37].

The test results showed that all isolates belonging to the species *C.albicans* form the germ tube when incubated at 37°C for 3 hours in 0.5 ml of human serum, while there was no germ tube formation from the other non-albicans species (*C.tropicalis, C.parapsilosis, T.glabrata*) under the same conditions.

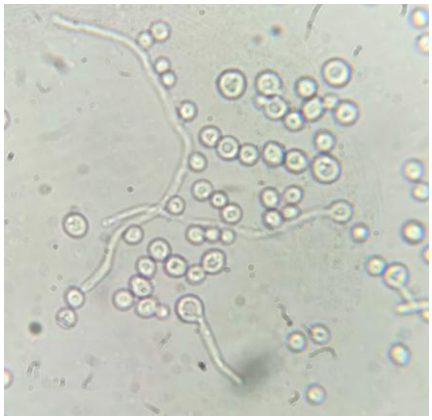

Figure 6: Germ tube of *C. albicans* in human serum at 37C° for 3hr (40X).

Adhesion test

Adhesion is regarded as the first stage in the progression of infection to host tissues, and it is a prominent virulence factor described for *Candida* and yeast-like fungi of medicinal interest. Furthermore, *Candida* is specialized to cling to human epithelial cells via hydrophobic interactions between yeast cells and the epithelial cell surfaces [38.39].

All the isolates of *C. albicans* 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.

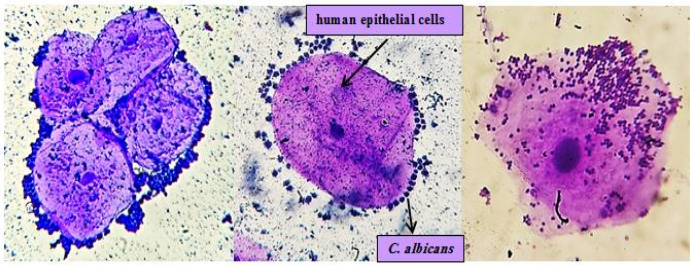

Figure 7: Adherence of *C. albicans* attached to human epithelial cells.

Biofilm formation on congo red agar

In order to perform the biofilm formation experiment, *C. albicans* isolates were grown on congo red agar (CRA) for 24 to 48 hours at 37°C. Dark red colonies turned into black, dry, crystalline colonies, indicating a successful outcome for robust biofilm production. Isolates with poor biofilm makers occasionally turned pink, but their colonies' cores did occasionally darken. Colonies produced by biofilm-negative strains were either very light pink or white in appearance[40].

The test results showed that from 64 *C. albicans* isolates, there were 16 (25%) strong biofilm producers, 6 (14.28%) were considered as weak biofilm producers

and the remained isolates were negative results were shown by colonies with a white or extremely pale pink color.

Strong biofilms formed by *C. albicans* isolates can be attributed to their genetic capacity to attach, generate a dense extracellular matrix, and form highly organized biofilms made up of many cell types encased in extracellular matrix[41,42,43].

Table 3: biofilm strength levels in *C. albicans* isolates with the type of clinical specimens

Type of specimens	Strong biofilms	Weak biofilm	Non-biofilm	Total
Urine	5(12.82%)	2(5.12%)	32(82.05)	39
Oral swab	11(9.82%)	4(3.57%)	97(86.6%)	112
Total	16(25%)	6(14.28%)	129(85.43%)	151

Figure 8: *C.albicans* growth on congo red agar (CRA) after incubated for 24 to 48 hours at 37 degrees Celsius. A positive result showed dark red colonies, weak biofilm producers remained pink. Negative results were shown by colonies with a white or extremely pale pink color.

Conclusion

According to the data obtained by this study, it concluded that there was a high prevalence of fungal infections caused by the pathogenic yeast species *C. albicans* isolates especially among adult female immunocompromised patients, and the studies described here form the basis for investigations into the mechanisms of *Candida* biofilm formation and provide the means to design new treatments for biofilm-based infections.

Acknowledgement

Thank you to all of the patients and families who cooperated and agreed to share some of their information for the purpose of conducting this research.

Conflict of interest

The authors declare no conflict of interest.

Ethics

All participants given informed consent to participate in the study and were assured that any information they provided would be used strictly for the purposes of this study and kept secret.

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