# Isolation and identification of candida albicans from children patient with candidiasis from Ramadi city, Iraq

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Abstract
*Candida albicans* are one of the members of a fungus that normally lives mucosal and can cause diseases in humans, cutaneous, mucosal, and disseminated invasive infections, especially in babies. The study aimed to isolate and identify Candida pp that caused Oral candidiasis in children in Ramadi city, Iraq. About 135 samples were collected from babies aged between one day and a year in both genders.The result of morphological and biochemical tests showed for pathogenic samples that among 90 isolates of Candida species, the predominant type was the Candida albicans which accounted for 39 isolates (%43.33), 24 samples from *C. tropicalis* (24. 66%), 15 samples from *C. krusie* (% 16.66) and 12 samples from C.*dublieninsis* (13.33 %).This study included the evaluation of *C. albicans* isolates, phospholipase enzyme production on egg yolk agar, and hemolytic activity on sugar-enriched sheep blood agar.

Keywords: *Candida spp*, *C. albicans*, Candidiasis, Oral Thrush

## 1. Introduction

The fungi are found in all environments and different types of symbiosis appear, there are more than (6000) diagnosed types of fungi, 600 of which are pathogenic to humans (Gibert et al., 2019). Candida sppnormal flora in healthy people is found in the mucous membranes such as the eyes, ears, sinuses, nose, mouth, digestive tracts, genitals, stool, skin, etc. It is beneficial to the body, but in the event of animbalance in the natural flora causes an increase in the growth of candida than the normal limit, causing a disease in humans called candidiasis,there are more than 150 species of Candida spp. But only 20 species cause human diseases, and Candida albicans is one of the most common fungal infections (Hani et al., 2015).

Candida yeasts consider this area as a natural flora of the mucous membranes in the body or a change in the external environment to a parasitic pathogenic fungus. In this case, the case is what is known as candidiasis.Studies indicate that Candida yeast colonies are naturally present in the oral cavity of 20-40% of healthy individuals and the respiratory tract, vagina, and gastrointestinal tract (Martins et al., 2014).

Oral candidiasis is a typical local opportunistic infection of the oral mucosa that is treatable. It occurs in children, the elderly, and people with cellular immune deficiency. The symptoms are limited by white plaques in the mouth, palate, and tongue(Sharma, 2019).Candida yeast in its normal state does not cause disease, but it is activated when the immune system is suppressed or an imbalance occurs in the normal microscopic balance, so C.albicans can transform into a pathogenic organism, as it grows excessively, leading to the occurrence of diabetes, surgical patients, infantile disease, and surgical patients AIDS(Sydnor and Perl, 2011).

Candida yeast possesses virulence factors and specific strategies that help it localize, cause disease, and overcome the host's defenses.It can grow in a variety of phenotypic forms ranging from Unicellular budding yeast (Blastospore), Pseudomycelium, and True hyphae.This transformation in shape is considered one of the most important virulence factors that help it invade tissues and escape from phagocyte cells. Other factors related to virulence include the production of hemolysin protein, the formation of the germination tube, the production of toxins, and the production of enzymes such as phospholipase and proteinase (Raut et al., 2017).

Recent decades have witnessed an increase in pathogenic fungal infections, which became one of the most prevalent diseases in developed countries. Oral
candidiasis reported in newborns and infants (Dantas et al., 2021). The most important diseases caused by Candida spp oral candidiasis, are systemic infection, and skin infection (Vainionpää et al., 2019).Therefore, the study aims to isolate and identify Candida spp associated with the oral cavity in children in Ramadi city, Iraq.

### Materials and working methods

A collection of 135 samples was collected from the

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mouth of children infected with candidiasis attending or Hospitalized patients at Hospital Obstetrician-gynaecologist, Al Ramadi in Iraq, whose ages ranged from 1 day – 1 year, from both sexes, males and females. For the period from 1- 11-2021 to 10-11-2022.Where the clinical examination was conducted for those who were reviewed by specialized doctors after diagnosing the injury, as samples were obtained from the patients by sterile cotton swabs containing physiological salt solution NaCl. The. Each patient was assigned a questionnaire containing some general information about the auditors.

Laboratory examinations of samples:

### 1- Direct Microscopical Examination

A drop was taken from the swab stick to be examined and placed on a slide and the cover of the slide was placed on it and passed over the flame by moving it over it two or three times, then it was examined by light microscopy under the force 10X and 40X to ensure the presence of yeasts and pseudomycelium **(Koneman and Roberts, 1985).**

### 2- Isolation and Purification

The swabs taken on solid Sabouraud medium (SDA) with chloramphenicol were grown in Petri dishes and incubated at 37 °C for 24-48 hours (Ellis, 1994). single colonies were isolated and purified from all samples previously cultured on SDAC solid Sabouraud medium for diagnostic testing (Marsh and Martin, 2009).

### Identification

#### 1- Characteristics Morphological

The morphology of colonies growing was examined and color, shape, texture, diameter, height, and odor were observed (Ellis et al., 2007).

### 2- Microscopic Characteristics

A portion of the colony was taken with the Inoculation loop and mixed with a drop of Lactophenol Blue Stain. Then the sample was spread on a sterile glass slide and covered with a Cover Slide. Then it was examined by light microscope under the power 10X and 40X to observe yeast cells pseudohyphae and blastoconidia. A second smear was taken on another sterile glass slide, stained with Gram stain, thenfixed on the fire flame and examined for sprouting (Ellis et al., 2007).

### Biochemical Tests

### 1- Growth test on Chrome Agar Candida

The test was conducted by taking a portion of the pure yeast colony by a sterile vector, aged 24 hours, developing on SDA medium and plotting it on Chrome Agar medium. The dishes were incubated at 37 °C for 24-48 hours. The strains were diagnosed according to the manufacturer's instructions. *Candida spp.* can be identified by the color and appearance of the colonies*c.albicans* is green and *c.tropicalis* is blue, *c. dublieninsis* is bluish-green in color, and*C.krusei* is dark pink**(Horvath et al., 2003).**

### 2- Germ Tube Forming Test

The tubes were inoculated with a portion of a pure colony of growing yeast on solid Sabouraud medium (SDA) and incubated at 37°C for 2-3 hours. Then a drop of the suspension was taken, placed on a sterile glass slide, and examined under a light microscope to view the germination tube. This examination is characteristic of *c. albicans*, as it is noted that the germ tube emerges in the form of a bud from one side of the cell 3-4 times the length of the cell itself **(Ellis et al., 2007)**.

### 3- Chlamydospores Forming Test

This test is considered one of the distinctive diagnostic characteristics of Candida. a single pure colony of yeast to be diagnosed was taken growing on SDA medium by means of a sterile inoculation needle without contact with the agar and inoculated with it in the middle of the corn flour agar (CMA) Agar Corn Meal. Then the dishes were incubated at 37 °C for 48 hours. A drop of lactophenol blue dye was sterilized and examined with a light microscope under 10X and 40X power to observe the presence or absence of Chlamydospoe (Marsh and Martin, 2009; Kangoga et al., 2011).

### 5- Testing the ability of Candida to grow at a temperature of 45° C

The method of **Pinjon et al, (1998)** was followed to test the ability of yeast to grow at a temperature of 45 °C, by plotting the samples on the Sabouraud dextrose agar SDA medium,then incubated at 45 °C for 48- 72 hours. This test is used to distinguish between yeasts that appear close to the SDA medium with the color *C.albicans*yeast-like yeast.

### 6- Test Sugar Fermentation

The test was done according to **Roberts, (1990)** method by adding 2 ml of the medium of the fermentation of sugars into test tubes in sterile container AED put in an inverted tube, it hasbeen added 2 ml of stockpiling sugar solution (glucose,sucrose, galactose, lactose, and maltose) and added drops of red phenol until the center has changed the color to red, then the tubes were inoculated with yeast suspension and incubated at a temperature of 30 ° C. The results were followed up daily for a period of time. 10 days. The evidence of positive test positivity is the change in the color of the medium from red to yellow and the formation of gas in the AED tubes.

### 7. Hemolysin production test

The test used to identify the positive result when hemolysis occurred around the colony by blood agar medium and the medium was inoculated with a part of the pure yeast colony to be tested, and the dishes were incubated at a temperature of 37°C for 18-24 hours (Levinson and Jawetz, 1996).

### 8. Proteinase Test

This test is done by transferring part of the young colony at the age of (18-42) hours to the milk medium and making pollen form spots. The incubated at a temperature of 35 °C for 1 hour, after which it can be investigated through the formation of a transparent area around the inoculation area, as this indicates the positivity of the test (Al - Dabagh, 2015).

## 2. Results and discussion

### Examination of Isolated Samples

The results showed direct microscopy and culture on Sabouraud dextrose agar medium for 135 swabs from the mouth of children infected with Candidiasis. The presence of candidiasis in only 90 samples (66.6%) of the total number of sick children was positive, and the total number of male children with oral thrush who showed a positive reaction to direct culture on SDA medium was 68 samples with a rate of (75.5%), while the number of female was 22 samples ( 24.5% ) as shown in Table (1).

**Table (2)** showed the results that there were significant differences in the level of birth (0.05) for children with oral candidiasis and according to the type of birth, where the highest incidence rate was 62 cases with natural childbirth, with a rate of (68.88%), while the infected children who were born by Caesarean section were 28, (31.11%). The infected children who depended on mixed feeding were 42 cases of infection (46.6%), artificial feeding was 33 cases (36.6%) and relied on breastfeeding 15 cases, with a rate of (16.6%).

The results showed that there are significant differences between the percentage of children infected with oral candidiasis who live in the countryside and those who live in cities at a significant level (0.05), as the number of infected children in the countryside was 71 cases (78.88%), while the infected children who lived in the city were 19 cases (21.11%). Another hand, the results showed that there were significant differences between the age groups of children with oral candidiasis, as the highest infection rate was recorded for the age group 6-9 months 30 cases with a rate of (33.33%), followed by the age group 3-6 months, 21 cases (23.33). %), while the lowest infection rate was recorded in preterm infants by 8 cases (8.33%) Table (3).

the results mentioned the relationship between infection with candida, the child's age, the type of breastfeeding, the place of living, and the sex of the child. The results referred to the probability of infection in males as more than that in females, and the infection of children from mixed feeding is the one that tops the list of conditions, followed by artificial feeding and breastfeeding.

The difference in results is due to the fact that this diagnosis depends on the experience of the physician, and there are some symptoms of diseases similar to candidiasis such as bacterial and viral infections in the mouth and diaper areas, or overlap between types of antibiotics as a result of excessive or indiscriminate use of these Antibiotics without consulting a specialist doctor. It can also be attributed to the inappropriateness of the development conditions for the nutritional media or the environmental conditions that were adopted during the study (Dewhirstet al., 2010).

The infection of candidiasis in the mouth and thighs of children is attributed to the fact that the candida is present as normal flora in the human body, which quickly turns into pathogenic opportunism when appropriate conditions are provided for it, such as lack of immunity and lack of interest in oral hygiene and the diaper area for children Increases the chance of contracting the disease (Bezerra et al., 2015).

Therefore, relying on clinical diagnosis only does not give sufficient information for the diagnosis, so the diagnosis must be made by other types, such as direct microscopy, which gave a more accurate result than the clinical examination, because this examination depends on the actual presence of yeast cells, fungal hyphae, or any part of the pathogen. (Jafarian et al., 2022).

Table 1: Number and percentages of male and female samples and their response to growth on S.D.A. medium

| Type of examination | Total number | Positive samples | Percentage | Negative samples | Percentage |
|---|---|---|---|---|---|
| **Meal** | 95 | 68 | 71.5% | 27 | 28.5% |
| Female | 40 | 22 | 55% | 18 | 45% |
| All number | 135 | 90 | | | |

Table (2): The results level of birth and breastfeeding for children with oral candidiasis.

| Type of Examination | Number of Infection | Percentage |
|---|---|---|
| Natural childbirth | 62 | 68.8% |
| Cesarean section | 28 | 31.2% |
| Mixed feeding | 42 | 46.66% |
| Artificial feeding | 33 | 36.66% |
| Breastfeeding | 15 | 16.66% |
| Chisquer =9.244a df = 2 P value= 0.010 | | |

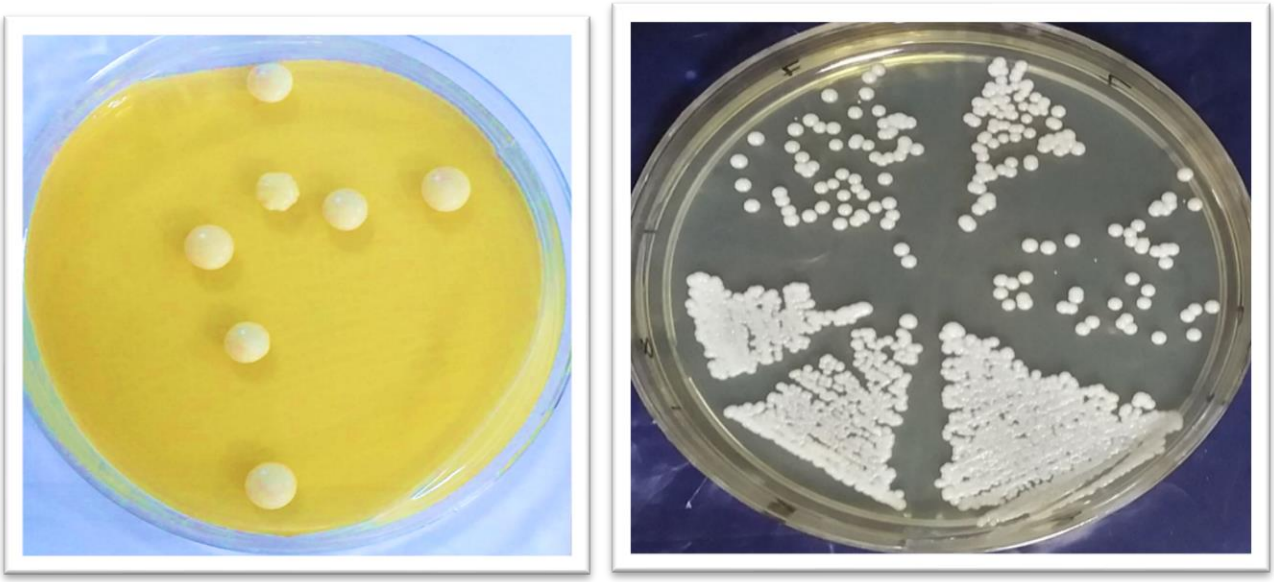
Table (3): The level of living and age categories for children with oral candidiasis.

| Type of Examination | Number of Infection | Percentage |
|---|---|---|
| City lives | 19 | 21.11% |
| Countryside lives | 71 | 78.88% |
| Preterm birth | 8 | 8.88% |
| 1-3 month | 11 | 12.22% |
| 3-6 month | 21 | 23.33% |
| 6-9 month | 30 | 33.33% |
| 9-12 month | 20 | 22.22% |
| Chi-squer = 17.000a df = 1-4 P value= 0.002 | | |

## Isolation and identification of yeast

The colonies grew on the medium an oval or spherical shape, white to creamy, round, Smooth to the touch, and convex in shape (Figur 1). This is results agree with **Garcia et al. (2020)** who also mentioned that the SDA medium is considered one of the typical media for isolating different types of yeasts. It is an essential medium for isolating Candida, as it allows its growth and prevents the development of many types of oral bacteria, due to the low pH of this medium.

Raju and Rajappa (2011) indicated that adding the antibacterial agent to the SDA medium increases the selectivity of this medium. SDA is a widely used method for the primary isolation of Candida spp. from clinical samples, in which Candida spp produces creamy, smooth, and convex colonies that may wrinkle when over-incubated (Kangogo et al., 2011).



*Figures (1): Growth of Candida on Sabouraud Dextrose Agar Medium*

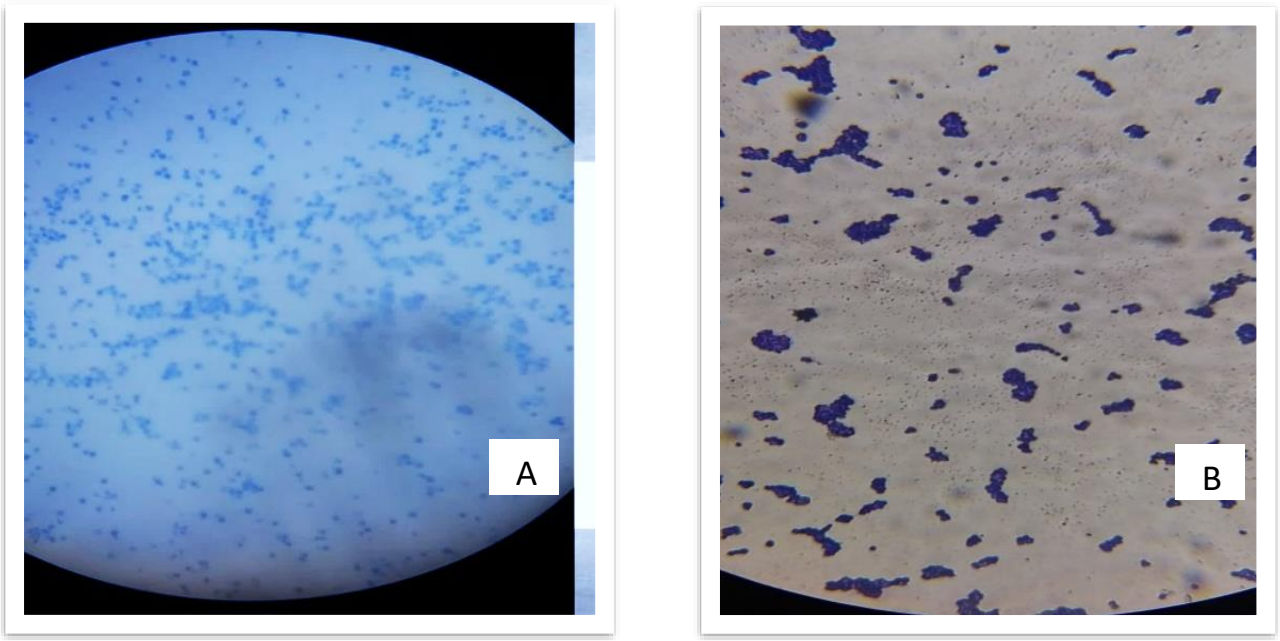



*Figure 2: Candida albicans yeast stained with (A) lactophenol blue dye and (B)gram stain(under 40Xmagnification).*

### Identification using differential media HiCrome Candida agar M1297A.

The results of this test showed the growth of all isolates on the differential Candida differential agar HiCrome medium at a rate of 100%, and the growing colonies appeared in different colors on the differential medium and according to the percentages of 39 isolates (34.3%) of c. albicans, in which the color of the growth of the colonies was light green. While 12 isolates (13.3%) belonged to C.dubliensis, whose colonies appeared in dark green color, 15 isolates (16.6%) belonged to C. krusei, whose colonies appeared in fluffy purple color, and 24 isolates (26.6%) belonged to C. tropicalis, whose colonies appeared in blue to purple Figure 3.

The discrepancy in the distribution of Candida spp found in several studies is due to the variance in the patient sample, diet, and antibiotics used (Arastehfar et al., 2019). Also, oral hygiene and the water plays an important role in the diversity of pathogens, the cause of which may be attributed to the dominance of c. albicans over other species to their virulence factors such as adhesion, biofilm formation, and secretion of degrading enzymes, as well as their ability to transform (Willis et al., 2018).

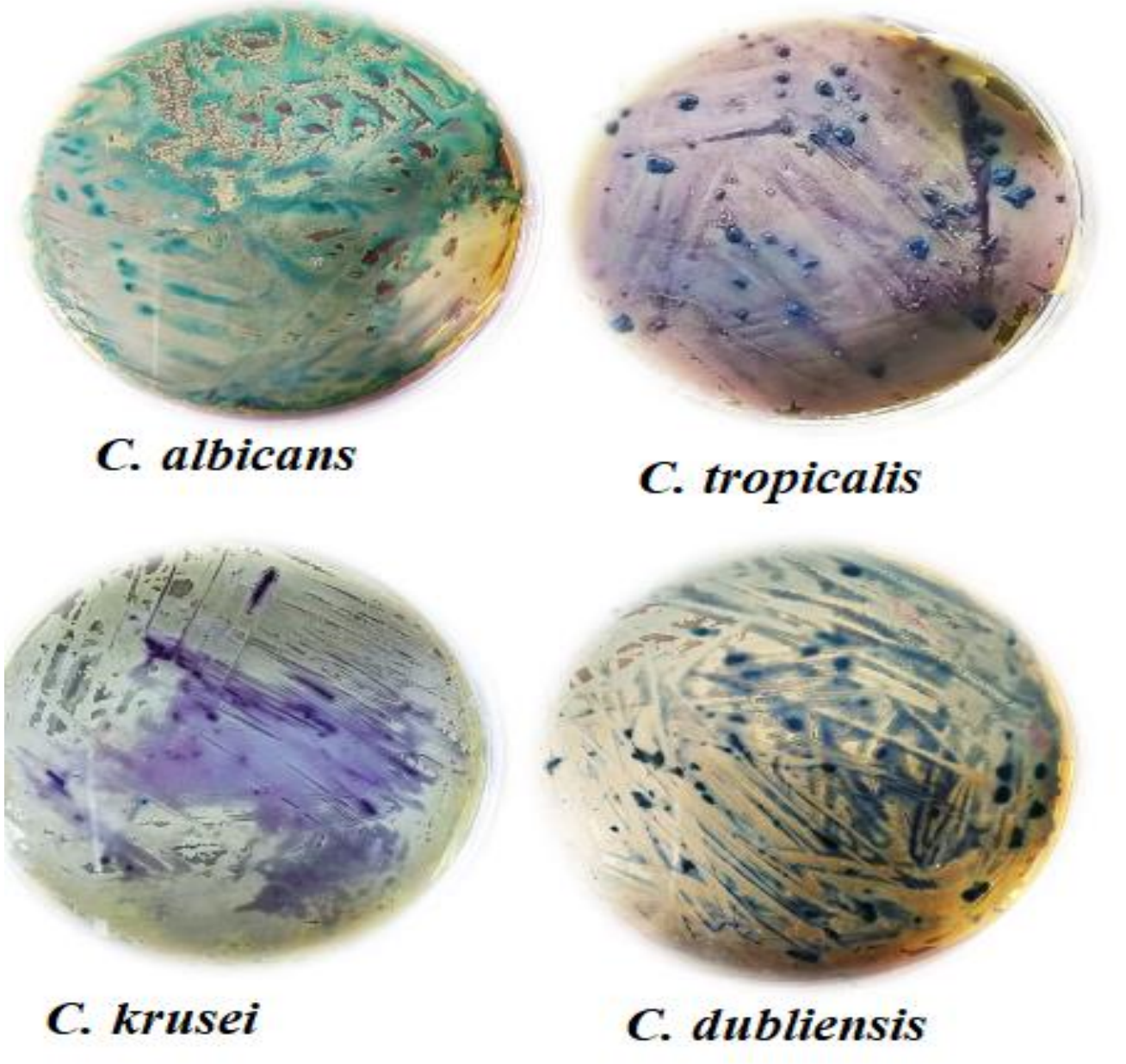



*Figure 3: Diagnosis of Candida spp. on differential media HiCrome Candida agar.*

### Biochemical Test for the diagnosis of Candida spp.

Table (4) showed some biological and biochemical tests of Candida spp for children with oral thrush. all types of Candida spp yeast showed a positive result for growth on an SDA medium at a temperature of 37 C for a period ranging between (72-48) hours, another hand, all types of Candida showed a negative result for the urease enzyme test. Also, the ability of yeast to form the filamentous and yeast form during the binary formation test after sowing on two plates of SDA medium and incubating them at two temperatures, the first 37 C and the second 25 C for a period of (24-48) hours, and the ability of c.albicans yeast to grow at a temperature of 45 ° C,

another hand, the yeast of c.albicans and c.dubliensis showed only the fermentation of sucrose. While, the yeast of c. albicans and c. tropicalis showed their ability to ferment maltose and lactose, and the ability of all types of yeast mentioned to ferment glucose and their inability to ferment the sugar lactose.

Where the positive result indicates a change in the color of the medium from red to yellow and the production of gas inside the AED tubes, and the negative result is no change in the color of the medium. As for lactose, the isolates belonging to this species were not able to ferment it (Table4).

We note through these results that all types of yeasts identified have the ability to ferment and metabolize glucose, as most species tend to isolate themselves from using glucose sugar as a simple food source rich in energy, such as c.albicans yeast and C. tropicalis yeast have a high ability to exploit most sugars and This explains its presence in high proportions among the isolated species. and it has been able to grow in high temperatures.

Table (4) shows the results of biochemical tests for the *candida spp*

| Tests | | Type | | | |
|---|---|---|---|---|---|
| | | *C.albicans* | *C.tropicalis* | *C.Krusei* | *C.dubliensis* |
| Growth in 37 c | | + | + | + | + |
| Urase | | - | - | - | - |
| Growth in 45 | | + | - | + | - |
| fermentation of sugars | Sucrose | + | - | - | + |
| | Glucose | + | + | + | + |
| | Lactose | - | - | - | - |
| | Maltose | + | + | - | - |
| | Galactose | + | + | - | - |

## Germ tube formation to C.albicans.

Figure 4 showed that isolates of C. albicans, can form the germ tube when incubated at a temperature of 37 ° C for 2-3 hours, another hand, all C. tropicalis, C. dubliensis, and C. krusei could not form the germ tube under the same conditions.

This percentage is consistent with Tauraet al. (2013) whose results showed that only c. albicans can form the germ tube and this test is a diagnostic advantage. It has the ability to form the germ tube. Guo et al. (2021) indicated that about 95% of c. albicans have the ability to form germ tubes, a feature shared by c.dubliensisand C.stellatoidea in this assay and in the presence of the activator (serum). Which works on its formation, and the formation of the germ tube is in the form of a long extension of the cell surface, which plays an important role in the process of penetrating the tissues and epithelial cells lining the body and growing inside it in the form of pseudopodia and reaching the bloodstream (Consolaro et al., 2018; Kornitzer, (2019).

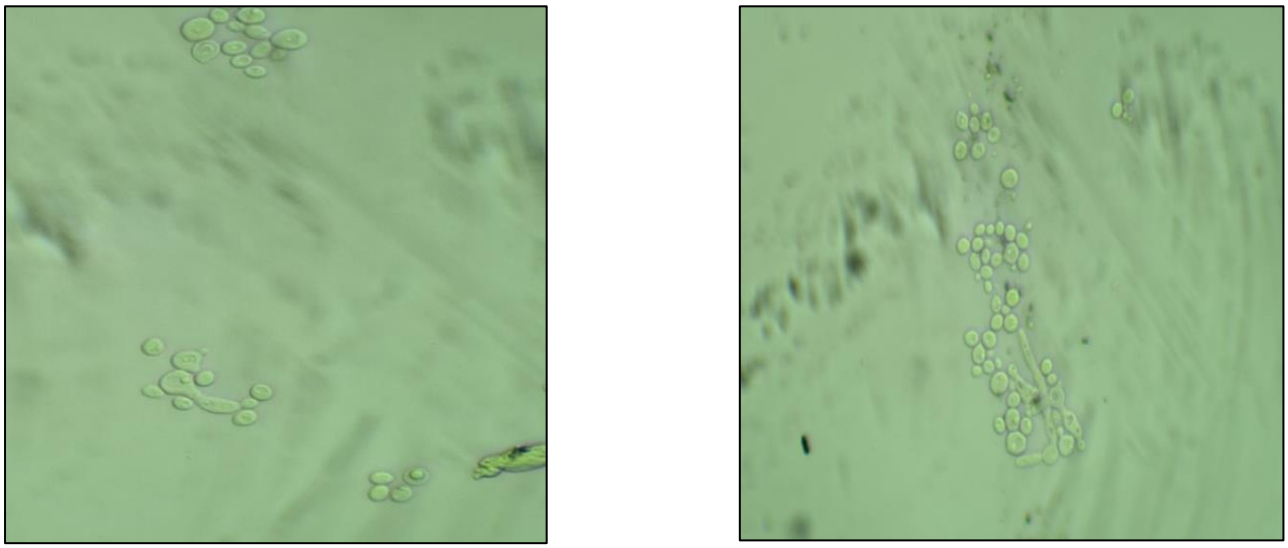


*Figure 4: Germ Tube Formation*

## The ability of Candida to the formation of Chlamydospores

The results showed that yeast c.albicans, c.tropicalis, and c.dubliensishad the ability to form Chlamydospores, while c.Krusei yeast could not put them in the same conditions. The formation of Chlamydospores is a diagnostic character of c. albicans. These spores are large in size with thick circular walls located at the end of the fungal hyphae. They may be single or grouped in groups when grown on a Corn meal agar (CMA) medium as a result of yeast starvation and lack of food sources in addition to unsuitable conditions for them, as this medium is described as starvation for yeasts (Mosaddad et al., 2019).This result is identical to that of Al-Hamdani et al. (2020) who found all the isolates

of c. albicans had the ability to form Chlamydospores, while other species did not develop it under the same conditions, and C. albicans, C. tropicalis and C. parapsilosis formed pseudo hyphae on CMA medium.

### The ability to produce the enzyme hemolysin

Table (5) shows the ability of Candida yeast to lysis of RBC by measuring the diameter of the halo zone formed on the medium compared with the colony diameter of children with oral thrush and all the samples examined showed a high ability to give positive results for hemolysis around the colony, especially in high glucose culture media, as shown in the Hemolytic Index (Hi).

Hemolysis by yeasts is a virulence factor and an important strategic process for the occurrence of pathogens and infections, as iron is liberated and is considered very important for growth and a catalyst for the vital functions of yeasts, and hemolysin production is positive in relation to glucose concentration and this is an indicator that people with diabetes are more susceptible to infection with candidiasis (Gomes et al., 2017).

Table 5: The intensity or strength of *C. albicans* yeast in producing the enzyme hemolysin.

| Hi | diameter of colony | Diameter of colonies | NO |
|---|---|---|---|
| 3.2 | 4.5cm | 2.0cm | A |
| 2.5 | 3.6cm | 2.3 cm | B |
| 2.8 | 4.5cm | 2.5cm | D |
| 2.6 | 4.0 cm | 2.5cm | E |
| 4.4 | 4.9cm | 2.1cm | F |
| 2.6 | 4.2cm | 2.6cm | G |
| 4.4 | 4.8cm | 2.5cm | H |
| 2.6 | 3.3cm | 2.0 cm | I |
| 2.7 | 3.1cm | 1.8cm | J |
| 2.7 | 4.0cm | 2.3cm | K |

### The ability of C. albicansto produce a Proteinase enzyme test.

**Table 6** shows the ability of *c. albicans* yeast to increase the sedimentation rate and the production of enzyme protease in milk medium, which constituted 39 isolates of oral isolates, with a percentage of 34.33%.

These observations are consistent with the results obtained by **Rorig et al. (2009)**who identified *C. albicans* as the largest enzyme producer, with neither C. glabrata nor C. parapsilosis revealing phospholipase and proteinase activities.Proteinase activities play essential roles in the pathogenesis of opportunistic fungi. The role of these hydrolytic enzymes in *c.albicans* and other yeast species appears to be related to the virulence of the species, and these enzymes' absence or decreased expression may indicate reduced virulence for some *Candida spp*(Ballal, 2008).

Table 6: Pz values for proteinase for different *C. albicans* and sites of origin

| Pz activity zone | Diameter sedimentation zone | Diameter of colonies | NO. |
|---|---|---|---|
| 3.0cm | 3.5cm | 1.7cm | A |
| 3.0cm | 4.0cm | 2.0cm | B |
| 2.2cm | 1.8cm | 1.5cm | D |
| 2.2cm | 2.5cm | 2.0cm | E |
| 4.6cm | 5.3cm | 2.0cm | F |
| 2.5cm | 3.0cm | 2.0cm | G |
| 4.4cm | 4.9cm | 2.0cm | H |
| 2.6cm | 2.0cm | 1.5cm | I |
| 3.0cm | 3.0cm | 1.5cm | J |
| 3.4cm | 3.6cm | 1.5cm | K |

## 3. Conclusion

The study focused on knowing the prevalence of oral thrush among infants in the city of Ramadi- Iraq. The results showed the presence of four types of yeasts belonging to the genus Candida, the yeast of C. albicans was the most common and considered the main cause of oral thrush.

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