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Diagnosis of yeasts isolated from the oral cavity and groin area in children of Kirkuk city/Iraq

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ABSTRACT

This study was conducted in the Graduate Studies Laboratory / College of Education for Pure Sciences - Kirkuk University; 200 swabs were collected from the oral and groin areas of children infected with candidiasis for both inpatients and outpatients at the Children's Hospital, the Maternity, Gynecology and Children's Hospital, and Azadi Teaching Hospital in Kirkuk governorate; whose ages ranged from 1 day - 3 years for both males and females; For a period ranging from February-2021 to March 25-2021. The immediate results of the microscopic examination showed that 152 smears (76%) were positive, while the results of culturing the smears on solid Sabouraud medium showed that 140 smears (70%) of the smears showed growth, and 60 smears (30%) of the smears showed no growth.

Five species belonging to the genus Candida were identified by biochemical tests and culture on Chrome Agar Candida medium. Led by the yeast *Candida albicans* with 74 isolates and (52.85%), followed by *Candida tropicalis* with 34 isolates and (24.28%), then *Candida lusitaniae* 13 isolates with a percentage of (9.28%), then *Candida krusei* with 11 isolates and (7.85%), while *Candida glabrata* recorded 8 isolates with a percentage of (5.71%).

Introduction

There is no environment devoid of fungi and different types of symbiosis appear, such as symbiosis, parasitism and predation, and there are more than (six thousand) diagnosed types of fungi, 600 of whichare pathogenic to humans [1], as they form yeast Candida spp. Normal flora in healthy people, where it 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 an imbalance in the natural flora, it causes an increase in the growth of candida than the normal limit, causing a disease in humans called candidiasis [2],

There are more than 150 species of Candida spp. But only 20 species of them cause human diseases,and *Candida albicans* is one of the most common types of fungal infections [3].

Recent decades have witnessed an increase in pathogenic fungal infections, which became one of the most prevalent diseases in developed countries [4]. Oral candidiasis reported in newborns and infants [5]. The most important diseases caused by Candida spp areoral candidiasis, systemic infection, and skin infection [6].

The study aims to:

1- Isolation and identification of Candida spp. associated with the oral cavity and groin area in children.

2- Diagnosis using Chrome Agar Candida (CAC)

Materials and working methods

Collection of Specimens

200 samples were collected from the mouth and diaper areas of children infected with candidiasis attending or sleeping in the Children's Hospital, the Maternity Hospital, Gynecology and Children's Hospital and Azadi Teaching Hospital in Kirkuk city, Kirkuk governorate center, whose ages ranged from 1day - 3 years, from both sexes, males and females. For the

period from February 9-2021 to March 25, 2021. 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 (Swabs Sterile Cotton) containing physiological salt solution NaCl. The samples were not infected to prevent mixing with the normal flora of the body. A questionnaire was assigned to each patient, which contained some general information about the auditors

Laboratory examinations of samples

Swabs taken from the mouth and diaper areas were examined in two ways:

1- Direct Microscopial Examination

A drop was taken from the swab stick to be examined and placed on a clean glass slide and the coverof the slide was placed on it and passed over the flame with moving it over it two or three times and then it was examined by light microscopy under the force 10X and then the force 40X to ensure the presence of yeasts and pseudomycelium [7]. A second glass slide, after fixation, was also stained with Gram-positive yeast cells [8] [9],and the sensitivity of the direct assay was measured by comparing the results of direct microscopy with the results of in vitro culture [10]. According to the following equation:-

Sensitivity = The number of positive cases x 100

Number of positive cases + number of false negative cases

2- Indirect examination

The swabs taken on solid Sabouraud medium (SDA) with chloramphenicol were grown in plastic dishes,and the cultured dishes were incubated at 37 °C for 24-48 hours [11].

Isolation and Purification

Individual colonies were isolated and purified from all samples previously cultured on SDAC solid Sabouraud medium for diagnostic testing [12].

Identification

1- Characteristics Morphological

The external appearance of colonies growing on SDAC was examined and the colony's colour, shape,texture, diameter, height and odor were observed [13].

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 and stained with Gram stain, thenfixed on the fire flame and examined for sprouting [13].

Biochemical Tests

1- Growth test on Chrome Agar Candida (CAC)

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 incubatedat 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, while *C.glabrata* is light pink to cream. Either *C.krusei* is dark pink. The yeast *C.lusitaniae* is pale pink to purple [14].

2- Germ Tube Forming Test (GTT)

To perform this test, a volume of 2 ml of egg whites (egg albumen) was taken and placed in sterile test tubes, then the tubes were inoculated with a portion of a pure colony of growing yeast on SDA solid Sabouraud medium and incubated at 37°C for 2-3 hours. Then a drop of the suspension was taken andplaced on a sterile glass slide and examined under a light microscope to view the germination tube. Thisexamination 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 [13].

3- Chlamydospores Forming Test

This test is considered one of the distinctive diagnostic characteristics of Candida. a single pure colonyof 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 by making three parallel lines on The surface of the agar in the dish, the length of the line is about 3.5 cm and the distance between one line and another is about 1.2 cm. A sterile glass slide cover was placed over the previous lines on the surface of the medium. Then the dishes were incubated at 37 °C for48 hours, after which the slide cover was lifted by sterile forceps and placed on a glass slide. 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 [12][15].

4- Surface Growth Test

The test was carried out by inoculating sterile test tubes containing Sabouraud sucrose broth (SSB) medium Inoculated with a portion of the pure yeast colony and incubating the dishes at a temperature of 37 °C for 24 hours. This test is used to observe the surface growth of Candidaspp [16].

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

The method of [17] 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 color *C.albicans* yeast like yeast.

6- Test Sugar Fermentation

The test was don according to [18] method by adding 2 ml of the medium of the fermentation of sugars into test tubes sterile container AED put an inverted tube, it has been 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 updaily for a period of time. 10 days. The evidence of positive test positivity is the change in the color of themedium from red to yellow and the formation of gas in the AED tubes.

7- Sugar Assimilation Test

The test was don according to [19] method after the preparation of the medium of sugars where they were cast in the Petri dishes and after solidification, 1 ml of yeast suspension was taken at the age of 24-48 hours and spread on the surface of the medium using a diffuser in the form of a letter L (L-Spreader) plates were left after inoculation for 30 minutes to dry, then holes were made with a diameter of 6 mm in the inoculated medium by means of a cork borer, and the solutions of storage sugars (glucose, sucrose, galactose, lactose and maltose) were added.

with a micro pipette and incubated at 30 °C for 2-4 days, and the dishes were followed during the incubation period to observe the presence or absence of yeast growth in the pits.

Results and discussion

Examination of Isolated Samples

Table 1 showed the results of direct microscopy and culture on Sabouraud dextrose agar medium for 200 swabs from the mouth and diaper areas of children infected with Candidiasis. The presence of candidiasis in only 152 children (76%) of the total number of sick children was positive, while the number of negative cases in the examination Direct microscopy 48 children, 24%.

Table 1: Numbers and percentages of laboratory examination of samples

Type of examination	Positive samples	Percentage	Negative samples	Percentage
Direct microscopy	152	%76	48	%24
In vitro culture	140	%70	60	%30

As for the positive results of laboratory culture, there were 140 children, (70%), while the number of Negative cases of laboratory culture 60 children, (30%). These results do not consistent with the findings of [20], where the infection was in direct microscopic examination by 40% and the infection bylaboratory culture was 50%, while it is consistent with the findings of [21], where 100 infections were collected from patients with oral candidiasis Oral, That were collected based on the clinical diagnosis of the specialized doctor, and the infection rate in direct microscopic examination was 96%, while the results oflaboratory culture were lower by 94%, and they also consistent with what [22] reached, wherethe percentage of infection in direct microscopic examination was 84%, while the results of laboratory culture It was 77% lower.

The difference in results in laboratory and clinical diagnosis is due to the fact that this diagnosis depends on the experience of doctors, and there are diseases similar to candidiasis in symptoms such asbacterial and viral infections in the mouth and diaper areas, or overlap between types of antibiotics as aresult of excessive or indiscriminate use of these Antibiotics without consulting a specialist doctor. It canalso be attributed to the inappropriateness of the development conditions for the nutritional media or the environmental conditions that were adopted during the study [23].

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. More precisely, [24] 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 a pathogenic opportunism when appropriate conditions are provided for it, such as lack of immunity and lack of interest in oral hygiene and the diaperarea for children Increases the chance of contracting the disease [25, 26].

Isolated Yeasts Diagnosis

The genus Candida spp. was diagnosed according to [13] based on microscopic, culturological, and biochemical characteristics, as well as confirmation of Candida diagnosis using the simple primary diagnosis Chrom Agar Candida (CAC) and secondary diagnosis using PCR technique. Candida spp. when grown on the common culture media, Sabouraud dextrose agar at a temperature of 37°C for 24-48 hours in the form of white to milky colonies, convex, smooth and glossy Figure 1 andcharacterized by its distinctive odor.

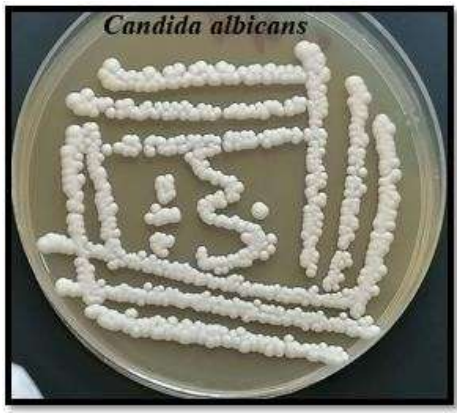

Fig. 1: Growth of *Candida albicans* isolate on SDA medium at 37°C

The colony was also examined microscopically after staining it with chromium and lactophenol blue dye, and the shape of the cells appeared spherical to oval and budding in Figure 2. This result was identical with [13, 27, 28, 22] Yeasts after staining with chromium dye appeared more clear when compared to lactophenol blue dye. The appearance of yeast cells stained blue due to the retentionof this dye by the Peptidoglycan layer in the cell wall [29], as the components of the outer cell wall contain multiple sugars. In the form of Glucans and Chitin, followed by Mannoproteins [30]. In addition, candida cells are Gram-positive [31].

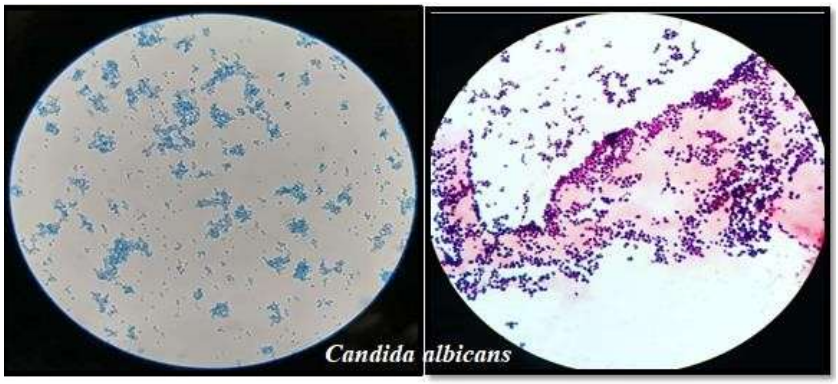

Fig. 2: *Candida albicans* yeast stained with chromium and lactophenol blue dye (under 40Xmagnification)

Diagnostic Using Medium Candida CHROM Agar (CAC)

The growth test on Candida CHROM Agar culture medium is one of the new and quick methods for diagnosing Candida species at the color level after planning the isolates on the medium and incubating them at a temperature of 37 °C for 24-48 hours. *C.albicans* is green, *C.tropicalis* is blue, *C.glabrata* is light pink to cream, either *C.krusei* is dark pink and *C.lusitaniae* is pale pink to purple. Figure 3 These results are consistent with that of [32, 33, 34, 22] aswell as matching the colors of the medium manufacturer.

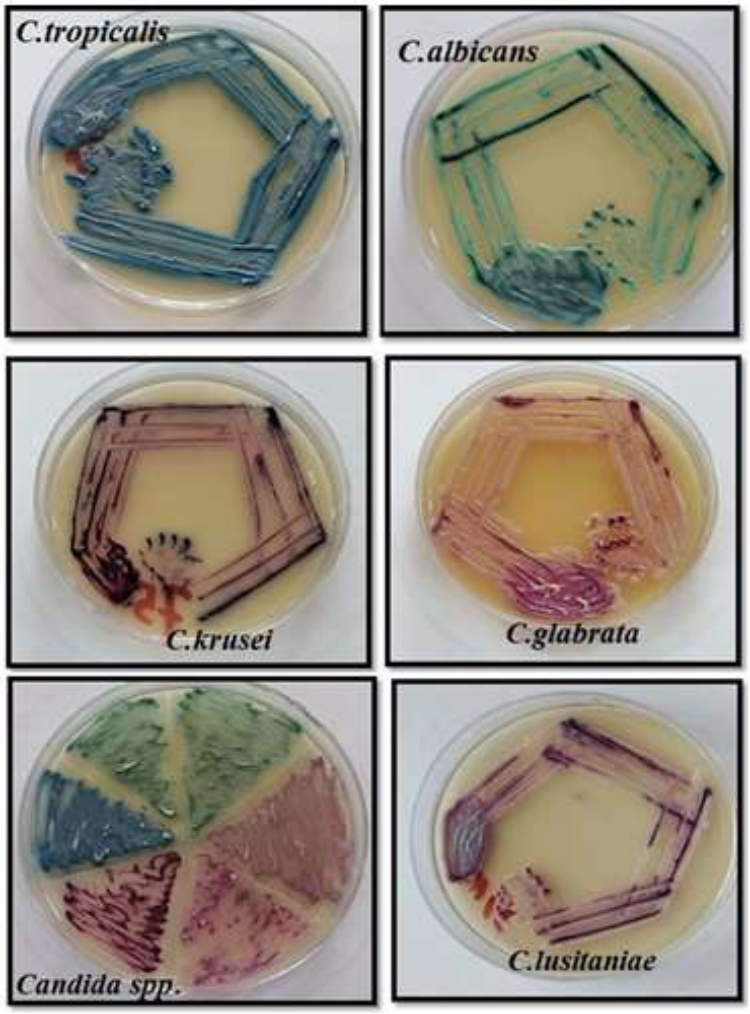

Figure 3: Diagnosis of Candida spp. On Chrom Agar (CAC

Table 2 indicates the species of Candida spp. isolated in this study, where five species of isolates were diagnosed from the total of 140 isolates of children infected with candidiasis, and the most frequent species was *Candida albicans*, where the number of isolates reached 74 isolates with a percentage of (52.85%), and this is the result It consistent with many studies that have been concerned with isolating oral candida, who ranked first in *Candida albicans* yeast compared to other species, and among these studies was what was reported by [21], where the number of isolates reached 52 isolates, at a rate of (69.3%), and it also consistent with the study of [22], as it reached the number of isolates is 49 with a rate of (63.64%), but it does not consistent with the study of [5], which did not record any infection with *C.albicans*, followed by *C.tropicalis*, *C.lusitaniae*, *C.krusei* and *C.glabrata*with a number of isolates of 34, 13, 11 and 8 isolates with a percentage of (24.28), (9.28), (7.85) and (5.71%), respectively. These results are consistent with the study of [35] who isolated several species, including the yeast *Candida albicans*, which was at the forefront, in addition to *C.parapsilosis*, *C.glabrata* and *C.tropicalis*. In the study of [36], the highest infection rate was recorded with *C.albicans*, 35 isolates, (92%), followed by *C.tropicalis*, 2 isolates, 5.26%, and finally *C.glabrata*, 1 isolate, (2.64%).

Several studies have indicated that the main cause of Candida infection, whether in the mouth or diaperarea, is *C.albicans*, followed by *C.glabrata*, in addition to other species of Candida in children and adults [37, 38, 28, 22].

Table 2: Number and percentage of presence of Candida species isolated during the study

Candida spp.	Number of species	Percentage of appearance
Candida albicans	74	52.85%
Candida tropicalis	34	24.28%
Candida glabrata	8	5.71%
Candida krusei	11	7.85%
Candida lusitaniae	13	9.28%
Total	**140**	**100%**

*Percentage of occurrence = number of isolates of each species ÷ total number x 100

The discrepancy in the distribution of candida species present in many studies is due to the variance in the sample of patients, the diet, and the antibiotics used

[39][40] indicated that oral hygiene and water used to clean it play an important role in diversifying pathogens, The reason for the dominance of *C.albicans* yeast over other species may be attributed to its virulence factors such as adhesion, biofilm formation, secretion of degrading enzymes, as well as its ability to transform [41].

Biochemical Test for the diagnosis of Candida spp.

1- susceptibility to Candida spp. On the composition of the germ tube

We note from Table 3 that all isolates of *C.albicans*, which constituted 49 isolates out of 92 oral isolates, with a percentage of 53.26%, while isolates of the diaper area formed 25 isolates out of 48 isolates, witha percentage of 52%, that formed the germ tube and this percentage consistent with what [28] mentioned who showed that all isolates of *C.albicans*, which constituted 44 isolates out of 70 oral isolates, equivalent to 62.85%, while isolates of the diaper area constituted 31 isolates out of 50 isolates, equivalent to 62% Figure 5, while isolates of species *C.tropicalis*, *C.glabrata*, *C.krusei* and *C.lusitaniae* did not form the germ tube under the same conditions and these results are in consistent with [42]whose results showed that only *C.albicans* had the ability to form Germ tube and this test was considered a diagnostic feature, and this result was similar to what [27] mentioned that only *C.albicans* has the ability to form the germ tube, and it also consistent with the study of [22], which showed that all *C.albicans* isolates formed the germ tube, while the dependent isolates For the species *C.*tropicalis, *C.parapsilosis* and *C.glabrata* did not form tubes under the same conditions, and [43] indicated that about 95% of *C.albicans* have the ability to form germ tube, and this feature is shared by *C.dubliniensis* and *C.stellatoidea* inthis assay and in the presence of the stimulator (serum). which works on its formation, and the formation of the germ tube, which is in the form of a long extension of the cell surface, plays an important role in the process of penetrating tissues and epithelial cells lining the body and growing inside them in the form of pseudohyphae and access to the bloodstream [29, 44].

Fig. 4: Germination tube formation in *Candida albicans* yeast (under 40X strength)

Table 3: Biochemical and physiological tests for the diagnosis of Candida species isolated during the study

Candida spp.	Characteristic of surface growth	Formation of pseudo hyphae	Formation of chlamydia spores	Formation of Germ tube	Growth at temperature	
Candida albicans	+	+	+	+	**37** °C	**45** °C
Candida tropicalis	–	+	–	–	+	+
Candida glabrata	–	–	–	–	+	–
Candida krusei	+	+	–	–	+	–
Candida lusitaniae	–	+	–	–	+	–

* + = test result is positive - = test result is negative

2- The susceptibility of Candida to the formation of Chlamydospores and pseudohyphae

Table 3 showed that all *C.albicans* isolates had the ability to form Chlamydospoers and Pseudohyphae by growing on Corn meal agar (CMA) and the result of this examination was identical to that of [28], which showed that all *C.albicans* isolates formed Chlamydospores and pseudohyphae, and the number of positive isolates was 43 out of 70 isolates of oral isolates. At a rate of 61.42%, the number of positive isolates for the diaper area was 30out of 50 isolates at a rate of 60%, and these results are in consistent with the study of [45]. This medium is a diagnostic characteristic of *C.albicans*, while the other species *C.tropicalis*, *C.glabrata*, *C.krusei* and *C.lusitaniae* were don't Chlamydospores under the same conditions, but it was found that *C.albicans*, *C.tropicalis*, *C.krusei* and *C.lusitaniae* formed pseudohyphae on the CMA medium and as This result is identical to what was found by [22], as all isolates of *C.albicans* had the ability to form Chlamydospores, while other species did not form it under the same conditions, and that *C.albicans*, *C.tropicalis* and *C.parapsilosis* formed pseudohyphae on CMA medium. The phenomenon of formation of Chlamydospores is a distinctive diagnostic characteristic of *C.albicans*. These spores are

characterized by being large in size with thick, circular walls located at the endof the fungal hyphae. They may be singular or grouped in clusters when Cultured on CMA medium as a result of starvation of yeasts and a deficiency in food sources as well as unsuitable conditions for them, where this medium is described as starvation of yeasts [12] Figure 5.

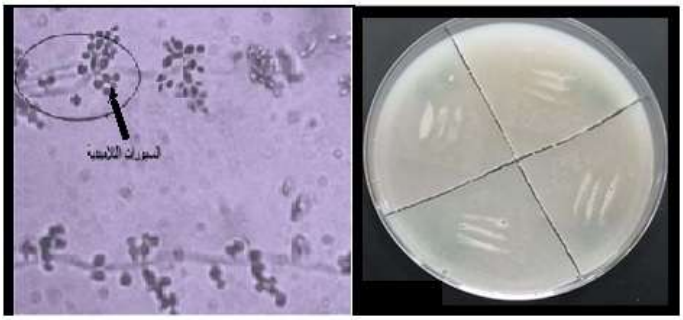

Fig. 5: Formation of Chlamydospores of *Candida albicans* on CMA medium under 40X

3- The ability of Candida spp. to grow surface

The surface growth results in Table 3 showed the ability of *C.albicans* and *C.krusei* to form creeping upward growth in the test tube containing the liquid Sabouraud medium sucrose SSB. This result is similarto that of [46] who demonstrated the ability of *C.tropicalis* and *C.krusei* develops a creeping upward growth on the wall of a test tube containing SSB liquid sucrose Sabouraud medium. Figure 6.

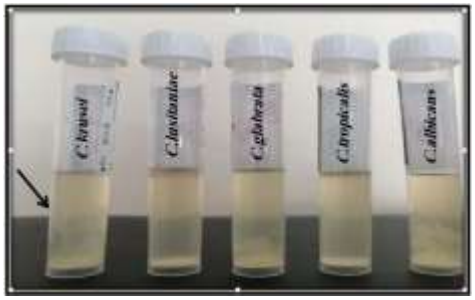

Fig. 6: Surface growth of *Candida albicans* and *Candida krusei* on SSB. Medium

4- Growth test at 45°C

Table 3 shows the growth test at 45 °C to distinguish *C.albicans* from other species of candida, and it was found that *C.albicans* is the only species that grows at 45 °C, while the other species *C.tropicalis*, *C.glabrata*, *C.krusei* and *C.lusitaniae* could not grow under the same conditions, and these results were consistent with the results of [28]. whose results showed that *C.albicans* yeast is the only species of candida that grows at a temperature of 45 ° C, as other species could not grow in the same conditions This result is also consistent with the findings of [47] who showed the ability of *C.albicans* yeast to grow at a temperature of 45 °C, which facilitated the process of distinguishing between *C.albicans* yeast and *C.dubliniensis* yeast, which cannot grow at the same temperature 45 °C. The reason for the ability of *C.albicans* yeast to grow at a temperature of 45 °C is due to its ability to withstand high temperatures because it is a high Thermophilic fungi fungus [17] Figure 7.

Fig. 7: Growth of Candida albicans on SDA medium at 45°C

5- The ability of Candida spp. to Ferment and Assimilate Sugars

Table 4 shows the results of the sugar fermentation test for Candida, 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. *C.albicans* yeast isolates showed their ability to ferment glucose, galactose and maltose, and their inability to ferment sucrose and lactose, while they were able to represent most of the sugars except for lactose, which it did not represent. This result consistent with the study of [22], which showed the ability of *C.albicans* yeast to ferment glucose, galactose and maltose. As for sucrose and lactose, the isolates belonging to this species were not able to ferment it in the Figure 8.

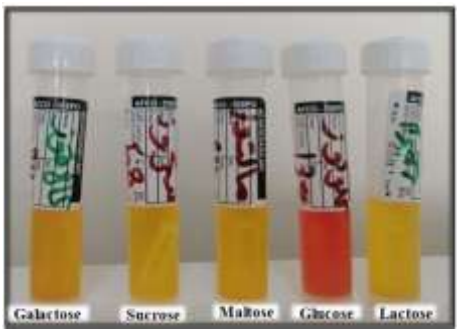

Fig. 8: Sugar fermentation patterns of Candida spp.

Table 4: Fermentation and representation of sugars for the diagnosis of isolated Candida species

Types of Yeasts	Sugar Assimilation						Sugar Fermentation				
	Mal	Lac	Suc	Gal	Glu		Mal	Lac	Suc	Gal	Glu
Candida albicans	+	-	V	+	+		+	-	-	V	+
Candida tropicalis	+	-	V	+	+		+	-	V	+	+
Candida glabrata	-	-	-	-	+		-	-	-	-	+
Candida krusei	-	-	-	-	+		-	-	-	-	+
Candida lusitaniae	+	-	+	+	+		V	-	V	V	+

1: Glucose 2: Galactose 3: Sucrose 4: Lactose 5: Maltose

(+) Test result is positive (-) Test result is negative (V) Test result is changed (+ , -)

Isolates belonging to *C.tropicalis* were able to ferment and represent glucose, galactose, sucrose and maltose but did not ferment and represent lactose, while isolates of *C. glabrata*and *C.krusei fermented* and represented glucose only and not fermented and represented both galactose, sucrose, lactose and maltose. All isolates of *C.lusitaniae* ferment and represent glucose, galactose, sucrose, and maltose, except for lactose, which *C.lusitaniae* was unable to ferment and represent. We note through these results that all species of diagnosed yeasts have the ability to ferment and represent glucose, as most species tend to Isolated to the use of glucose sugar as a simple food source rich in energy, as the yeast *C.albicans* and *C.tropicalis* have a high ability to exploit most sugars and this explains theirpresence in high proportions among the isolated species and this result consistent with both [13, 46, 22], who showed that the two species *C.albicans* and *C.tropicalis* possess a high efficiency in fermenting and representingmost types of sugars, and this is one of the reasons for their reliable presence when infected with Candidiasis.

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تشخيص الخمائر المعزولة من تجويف الفم ومنطقة الفخذ في اطفال مدينة كركوك/العراق

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الملخص

أجريت هذه الدراسة في مختبر الدراسات العليا / كلية التربية للعلوم الصرفة – جامعة كركوك؛ إذ تم جمع 200 مسحة من منطقتي الفم والفخذ للأطفال المصابين بداء المبيضات لكل من الأطفال الراقدين والمراجعين في مستشفى الأطفال ومستشفى الولادة والأمراض النسائية والأطفال ومستشفى آزادي التعليمي في محافظة كركوك؛ والذين تراوحت أعمارهم من يوم واحد– 3 سنوات لكل من الذكور والإناث؛ لمدة تراوحت من شباط –2021 إلى 25– آذار–2021. النتائج الفورية للفحص المجهري اظهرت وجود 152 مسحة (76%) نتيجة إيجابية.

فقد تم تشخيص خمسة أنواع تابعة لجنس المبيضات بواسطة الاختبارات البيوكيميائية والزرع على وسط كروم أكار كانديدا Chrome Agar Candida. جاءت في مقدمتها الخميرة *Candida albicans* بعدد 74 عزلة وبنسبة (52.85%) ويليه النوع 34 *Candida tropicalis* عزلة وبنسبة (24.28%)، ثم النوع 13 *Candida lusitaniae* عزلة وبنسبة (9.28%)، ثم النوع 11 *Candida krusei* عزلة وبنسبة (7.85%)، بينما سجل النوع 8 *Candida glabrata* عزلات وبنسبة (5.71%).