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Detection Of Some Virulence Factors Of Fungi Caused Otomycosis Isolated From Some Hospitals And Clinics In Mosul/ Iraq

Nabeel Taha Younes

University of Mosul / College of Science / Biology Department, Mosul-Iraq, nabeeltaha27@gmail.com

Muhammed A. Al-Kataan

University of Mosul / College of Science / Biology Department, Mosul-Iraq, moh1977729@gmail.com

Maha A. Al-Rejaboo

University of Mosul / College of Science / Biology Department, Mosul-Iraq, mahaalrejaboo2@uomsul.edu.iq[http](http://uomsul.edu.iq)

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Detection of some virulence factors of fungi caused Otomycosis isolated from some hospitals and clinics in Mosul/Iraq

<i>Authors Names</i>	ABSTRACT
<p>a. Nabeel T. Younes^a b. Muhammad A. Al-Kataan c. Maha A. Al-Rejaboo</p> <p>Article History Received on: 30/6 /2021 Revised on: 22 /7/2021 Accepted on: 25 / 7/ 2021</p> <p>Keywords: Otomycosis, Candida, Aspergillus, Mosul a new line</p> <p>DOI: https://doi.org/10.29350/jops.2021.26.4.1413</p>	<p>Otomycosis is a fungal infection that frequently involves the external auditory canal. In this study, we aimed to isolation and identification the fungal isolates as etiological agents of Otomycosis from some hospitals and clinics in Mosul with detection of their virulence factors of etiological agents. Positive fungal infection was found in (43) spicemens (71.6%). The most common fungal pathogens were <i>Candida</i> and <i>Aspergillus</i> species, with <i>C. parapsilosis</i> being the predominant isolates in (11) specimens (16.6%). Otomycosis was more common in Female in (26) specimens (43.3%). Otomycosis was the highest prevalence aged group 15-40 years (19) specimens (31.3%). The present study of virulence factors revealed that the highest biofilm formation isolates were <i>C. parapsilosis</i> is (10) isolates which were distributed between (2) strong and (8) weak biofilm formation. Where <i>C. trypicales</i>, was recorded as least isolates for biofilm production.</p>

1. Introduction

Fungi are among the most important living organisms spread in most environments, divided into molds and yeast. Molds are fungi that grow in the form of multicellular filaments called Hyphae. It reproduces by forming spores, one of the most famous of them *Aspergillus* and *Penicillium spp*, Yeasts are unicellular and reproduce by budding, there are several types of it, some of which are beneficial to humans and others are pathological, such as *Candida spp*. It is considered one of the most prevalent genera of yeasts due to its possession of several virulence factors, as well as its ability to cause infections in the ear, respiratory and digestive canals, and the urinary and genital tracts, as well as its entry into some cases into the bloodstream [6,20].

About 150 *Candida* species have been recorded, including 20 human pathogens; the common species cause more than 90% of *Candida* infections, namely: (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*). *Candida spp.* is a part of the normal flora of the human body as it resides in various anatomical sites in the human body such as External Auditory Canal (EAC) [8,2,32]. There are different conventional methods of Identification *Candida spp.* such as cultured on Sabouraud Dextrose Agar (SDA), Corn Meal Agar (CMA), Germ tube test to get a rapid identification.

^a University of Mosul / College of Science / Biology Department, Mosul-Iraq, E-Mail: nabeeltaha27@gmail.com

^{b,c} University of Mosul / College of Science / Biology Department, Mosul-Iraq, E-Mail: moh1977729@gmail.com; mahaalrejaboo2@uomsul.edu.iq

for *Candida spp.* CHROMagar Candida medium is used as a differential medium that prevents the growth of any microorganisms except *Candida spp.* [27,12]. The components of this medium help to identify *Candida* isolates by the color and appearance of the colony. The colony appears in special colors on the surface of CHROMagar medium as shown in table (5). The colony appears in these colors because of the basic substance of this media, which interacts with the enzymes that produce it by *Candida spp.* So, the GHROMagar medium possesses the ability to distinguish between *Candida* species itself and other yeast infections. [27].

Otomycosis is a fungal infection often located in the medial aspect of the external ear canal [12]. This infection may affect one or both ears. The main risk factors for Otomycosis include moisture, minor inflammation, the use of broad-spectrum antibiotics, steroids, chemotherapeutic agents, topical ear drops, physical injury, living in warm and humid climates, foreign objects in the ear canal (earplugs, earphones), the anatomy of the ear canal acts as a route and residence of fungal elements and frequent bathing or swimming. [5,17]. Data regarding Otomycosis epidemiology are scant in the relevant literature. There are no large epidemiological studies, thus the perspective about fungal distribution is based on data from sporadically conducted studies. A lot of them are done in Middle East countries, namely Iran [16], and two genera most frequently associated with Otomycosis are *Aspergillus spp.* and *Candida spp.* [33]. This study aims to isolation of fungal species that cause infection of the External Auditory Canal (EAC Otomycosis) and identification with modern and accurate methods, and detection some virulence factors of etiological agents.

2. Materials and Methods:

2.1 Preparation of ready cultures media:

Sabouraud Dextrose Agar (SDA).

The medium was prepared according to the instructions of the equipped company by dissolving 65 g of the medium powder in a liter of distilled water, then sterilized with Autoclave at 121 C° for 15 min. then cooled and then add 0.05 mg/ml of chloramphenicol to prevent bacterial growth Pour this medium into sterilized Petri dishes and store until use [3].

Candida CHROMagar medium :

CHROMagar medium was prepared according to the instructions of the processed company, with a weight of 45.9 g of the prepared medium, it was placed in a beaker and dissolved in 1000 ml of distilled water by heating, and it was boiled for one minute to help mix the substance, then cool the medium and pour it into sterilized dishes. This medium was used to differential *Candida* yeasts according to the color of the colony [11].

2.2 Collection of specimens:

Sixty specimens were collected from patients who visited Mosul General Hospital and the Republican Hospital in Mosul (ear, nose and throat department) and some ENT clinics on both sides of Mosul, right and left, during the period from September 2020 to November 2020, Where sterile cotton swabs were taken from the external auditory canal of the patients, and informational data related to the patients such as age, gender and other data were recorded.

2.3 Isolation and Identification

All ear swabs were cultured on Sabouraud Dextrose Agar (SDA) with duplicate, the first plate was incubated at 37°C and the other at 27°C for 7-10 with daily examination until the colonies appeared or revealed no growth.

All fungal isolates were stored in SDA slants as pure isolates in addition to maintain with glycerol 20% for yeast isolates and 10% for other filamentous fungi [2].

Filamentous fungal isolates were identified according to morphological characters and microscopic appearance [7,19], whereas yeast isolates were identified based on phenotypic features on CHROM agar [25] and API Candida system [31,34], in addition to evaluation of germ tube formation for *Candida* identification [15].

2.4 Virulence factor test:

Germ Tube Test

This assay was used to differentiate between *Candida* species, However, *Candida albicans* and *C. dubliniensis* only give positive results for this test in comparison with other species. The test was determined by using a 0.5 ml serum tube that inoculated with a small portion of young yeast colony and incubated at 37 °C for 2-4 hrs., then a drop of mixture was examined under a light microscope to show germ tube formation. [7,10].

Biofilm formation test

Congo Red Agar Method:

The alternative method described the screening of isolates for biofilm formation is Congo red agar containing Brain Heart Infusion (BHI) broth supplemented with 5% and Congo red. The medium was composed of BHI (37 g/L), sucrose (50 g/L), Agar (20 g/L) and Congo red stain (0.8 g/L). Congo red was prepared separately as concentrated aqueous solution and autoclaved at 121 °C for 15 min. and added separately when the agar has cooled to 55 °C, plates were inoculated and incubated aerobically at 37 °C for 2-3 days. Positive results were indicated by black colonies with a dry crystalline consistency. A non- biofilm producer usually remains pink. The experiments were performed in triplicate and repeated three times. [14].

3. Results and Discussion

3.1 Isolation and Identification of the fungi causing Otomycosis:

The results of collecting 60 specimens from the External Auditory Canal (EAC) of the ear for male and female patients with ear infections for age groups ranging from 2-70 years showed the presence of 43(71%) pathological isolates of *Candida* and *Aspergillus* species, *Candida* Otomycosis (21/41.6%) infection was found to be more prevalence. (Table 1). The dominant *Candida* species was *C.parapsilosis*, (Table 5). *Aspergillus* Otomycosis infections (11/18.3%) (Table 2). Dominant was *A. niger* and combined (*Candida* and *Aspergillus*) infection was recorded in far fewer number of subject with Otomycosis (7/11.6%). (Table 3).

In total, 43 cases of inflammation of the external ear canal (EAC) were recorded out of a total of 60 specimens of various fungal causes, and the total infection rate was about 71.6%.

Table (1): Prevalence of Otomycosis caused by infection from *candida* spp. Isolated from (EAC).

Clinical Specimens	Number of sample tested	positive cases	Percentage %	Total Samples
External Auditory Canal (EAC)	60	25	41.6	60

Table (2): Prevalence of Otomycosis caused by infection from *Aspergillus* spp. Isolated from (EAC).

Clinical Specimens	Number of sample tested	positive cases	Percentage %	Total Samples
External Auditory Canal (EAC)	60	11	18.3	60

Table (3): Prevalence of Otomycosis caused by infection from. *Candida-Aspergillus* Otomycosis Isolated from (EAC).

Clinical Specimens	Number of sample tested	positive cases	Percentage %	Total Samples
External Auditory Canal (EAC)	60	7	11.6	60

3.2 Age and Sex Distribution:

The results of collecting 60 specimens from patients with Otomycosis (EAC) showed the presence of 43 clinical cases of different types of yeasts and molds represented by *Candida spp.* and *Aspergillus spp.*, where the infection reached about 71.6% for both males and females and for different age groups as for the classification of specimens and according to the pathogen for each of the Sex and Age groups, they are shown in the (Table 4).

Infections with *Candida spp.* 25 cases of the disease, of which (15) cases are for Females, at a percentage of 25%, compared to 10 cases for Males, at a percentage of 10% of the total specimens.

As for the infection with *Aspergillus spp.*, the total number of infections was about 11, of which 7 were in Females, representing 11.6% of the total number of infections, and 4 in Males, at 6.6%. Combined (*Candida-Aspergillus*) infection was record in a far fewer number of the subject with Otomycosis. The total number of these infections were 7 infections Of which 3 cases are for females (5%), and 4 cases for males, representing (6.6%) of the total number of infections (Table 1). These results are in agreement with the findings [26,17].

As for the classification of infections by age groups (Table 4), the results showed that the age group 15-40 years was the most affected by the infection of the EAC in 19 cases with a percentage of 31.6%, followed by the age group 41-60 years with a rate of (18.3%), As for the age group under 14 years it recorded (15%) infection, and finally, the age group over 60 years old recorded the least cases, and there were 4 clinical disease cases with a rate of 6.6%. (Table 4).

The results showed that the age group between 15-40 years had the highest infections for all etiologic agents, whether the causative was yeast or molds, while the age group above 60 showed the lowest cases of all pathogens, and the age group under 14 years did not record combined infections (*Candida-Aspergillus*) Otomycosis.

As for the age group between 41-60 years, 6 infections were caused by *Candida spp.* And two cases were caused by *Aspergillus spp.*, while this age group recorded 3 combined infections (*Candida-Aspergillus*) Otomycosis. (Table 4). The results of this study were in agreement with what was mentioned by each of the [2,29].

Table (4): Prevalence of Otomycosis according to gender and age.

	<i>Candida</i> Otomycosis		<i>Aspergillus</i> Otomycosis		<i>Candida Aspergillus</i> Otomycosis		Otomycosis	
	No. Positive of total No.	%	No. Positive of total No.	%	No. Positive of total No.	%	No. Positive of total No.	%
Gender								
Male	10	16.6	4	6.6	3	5	17	28.3
Female	15	25	7	11.6	4	6.6	26	43.3
Age								
<14	7	11.6	2	3.3	0	0	9	15
15-40	9	15	7	11.6	3	5	19	31.6
41-60	6	10	2	3.3	3	5	11	18.3
60+	3	5*	0	0	1	1.6	4	6.6

* $p < 0.05$ consider as significant

3.3 Identification of causative agents

Identification of yeast(*Candida spp.*)

Cultural characteristics

Colonies of *Candida* species on SDA medium at 37°C form oval to the ovate round shape, convex with white to cream color and are smooth with a characteristic yeast smell. The *Candida* colonies in this study had the same characteristics mentioned.(Fig. 1). These results are in agreement with the findings of both [28,1].



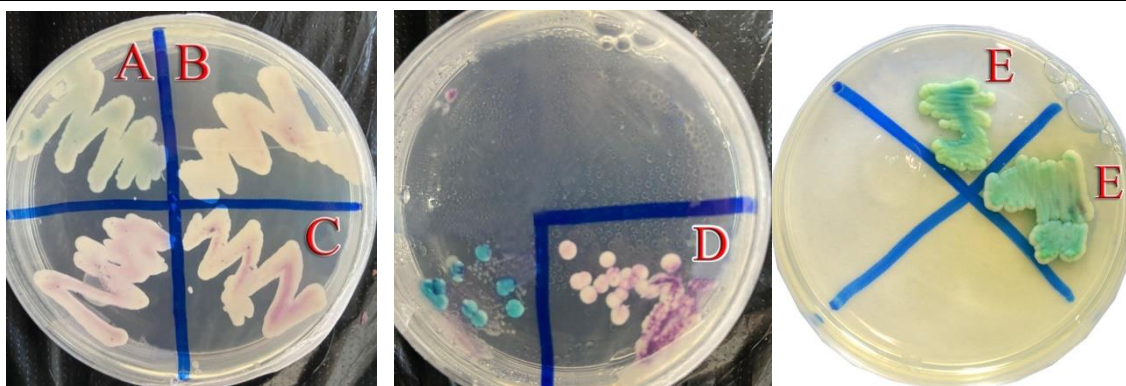
Fig. 1: *Candida spp.* Isolated on SDA medium

Identification on CHROMagar *Candida* media

Isolated yeasts(*Candida spp.*) were identified by culturing them on the differential medium (CHROMagar *Candida* Media). It appeared *C. albicans* in light smooth green colonies, while appeared *C.parapsilosis* in white to pale pink colonies, As for *C.tropicales*, it appeared in a steel blue colonies, while *C.krusei* appeared in purple-pink colonies, As for *C.glabrata*, it appeared in light white-purple colonies.[25] .(Table 5), (Fig. 2).

Table (5): Identification of *Candida* species by using CHROMagar media.

<i>Candida species</i>	Colony Characteristics CHROMagar medium	No. of isolates total (60)	Percentage (%)
<i>Candida albicans</i>	Light smooth green colonies	4	6.6
<i>Candida glabrata</i>	Light white-purple colonies	4	6.6
<i>Candida tropicales</i>	Steel Blue colonies	1	1.6
<i>Candida krusei</i>	Purpel-Pink colonies	5	8.3
<i>Candida parapsilosis</i>	White to pale pink colonies	11	18.3



- A: *C. albicans*
- B: *C. parapsilosis*
- C: *C. glabrata*
- D: *C. krusei*
- E: *C. tropicales*

Fig 2: CHROMagar showing different species of *Candida spp.*

Biochemical identification by API system.

The result of the API system was seen in table (6) which revealed the identification of 11 isolates as *C. parapsilosis*, five isolates as *C. krusei* , while *C. albicans*, and *C. glabrata* were identified in four isolates for each one and one isolate was identified as *C. tropicalis*.

Table (6): Identification of 25 *candida spp.* Isolated with the API *Candida* system.

<i>Candida species</i>	Total No. of isolated	API candida
<i>Candida albicans</i>	4	4
<i>Candida glabrata</i>	4	4
<i>Candida tropicales</i>	1	1
<i>Candida krusei</i>	5	5
<i>Candida parapsilosis</i>	11	11



Fig3: API *Candida* system

Identification of molds (*Aspergillus* species)

Isolation of molds was performed by inoculation of specimens on Sabouraud Dextrose Agar (SDA), which were incubated at 27°C for up to 7–10 days. Genus and species were identified based on macroscopic (features, appearance and color of colonies) and microscopic (*Aspergillus* species type of sporulation, presence of conidiophore, and radial phialids) characteristics. [19].

In the group of isolated yeast (*Candida spp.*) 25 strains were identified to the species level, Dominant was *C.parapsilosis* (18.3%), followed by *C.krusei* (8.3%), *C.albicans*, *C.glabrata* (6.6%), and *C.tropicales* (1.6%). (Table 5,6). Among 11 identified *Aspergillus* molds predominant were *A. niger* (16.6%) specimens followed by *A.flavus* (1.6%). (Table 2), (Fig. 4).

The results of this study were in agreement with what was mentioned by each of the [13,8,32,9]

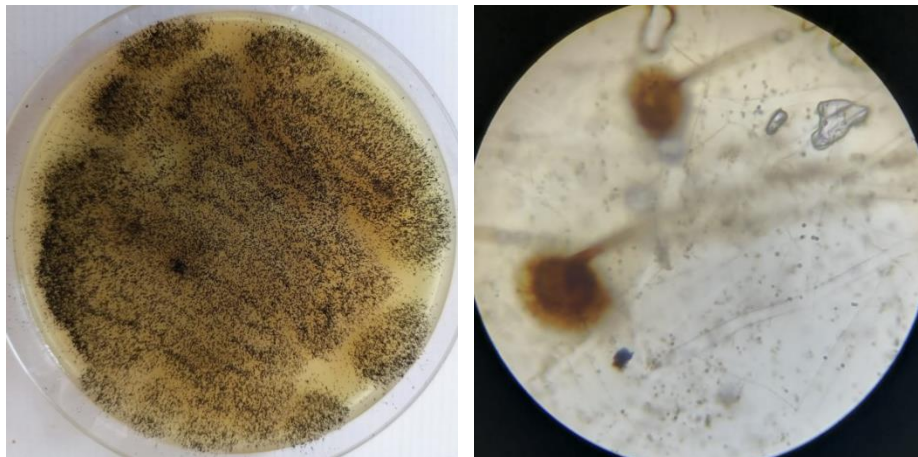


Fig.4: A: *A. niger* isolated on SDA medium; B: Microscopic features of *A. niger* (40X)

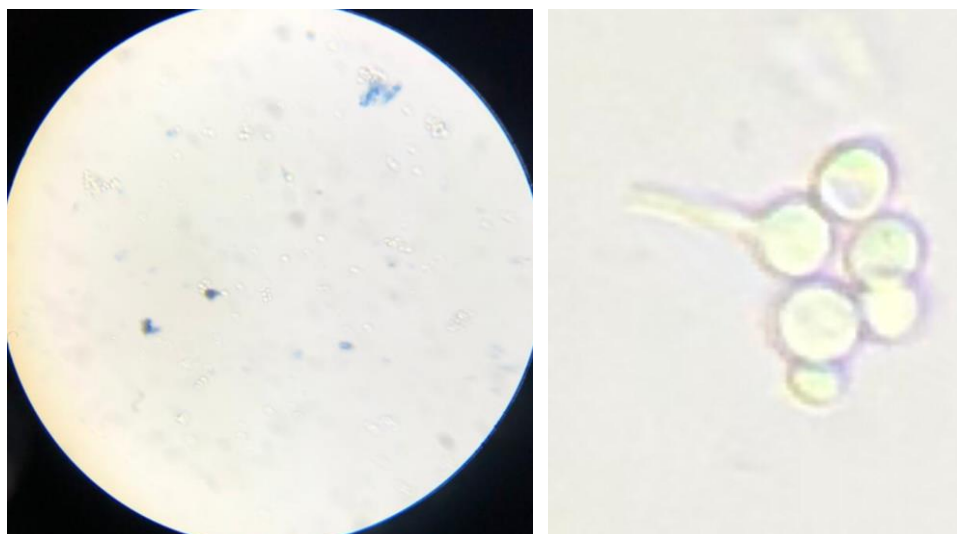
3.4 Detection of some virulence factors for *Candida* species

Germ tube formation test

The results of this test showed the ability of *C.albicans* yeast to form a germ tube after placing it in a tube containing blood serum for a 2-4 hours. According to the results showed, this type produced a germ tube and this is an identifying characteristic for this species only, and the other species did not appear in this study of the composition of the germ tube. This result was in agreement with each of the [15,23] which mentioned that *C.albicans* has the can form a germination tube. This germ tube is formed due to the presence of stimulating substances in the serum [24]. (Fig. 5), (Table 7).

Table (7): Identification of *Candida* species by germ tube formation.

<i>Candida species</i>	Total	Germtube Formation	
		Positive	Negative
<i>Candida albicans</i>	4	4	0
<i>Candida glabrata</i>	4	0	4
<i>Candida tropicales</i>	1	0	1
<i>Candida krusei</i>	5	0	5
<i>Candida parapsilosis</i>	11	0	11

**Fig.5:** Germ tube formation in *C. albicans*.

Biofilm formation by Congo Red Method

Biofilm detection experiments showed the presence of 25 isolates of *Candida* of various mentioned species, which showed the presence of 21 positive and 4 negative biofilm isolates for patients with (EAC) inflammation of Female and Male.(Table 8).

Congo Red Agar was used to determine the ability of *Candida* species to form a biofilm, as it is a simple and reliable method for determining the biofilm, and there was a range in the results between the isolates in their biofilm production between strong and weak.(Table 8). Positive specimens were indicated by black colonies with a dry crystalline consistency. A non- biofilm producer usually remains pink.(Fig. 6). This is consistent with what many researchers have mentioned .[14,21,18].

Twenty-one isolates of various *Candida spp.* showed a positive result for the Congo Red Agar method, including 4 isolates of *C. albicans*, 2 isolates of *C. glabrata*, 1 isolate of *C. tropicales*, 4 isolates of *C. krusei*, and 10 isolates of *C. parapsilosis*. The number of weak isolates was 12 for their biofilm production (Table 8). 4 isolates of different yeasts showed no biofilm production out of a total of 25 isolates, including 2 isolates of *C.glabrata* out of a total of 4 isolates, 1 isolate of *C. krusei* out of a total of 5 isolates, and 1 isolate of *C. parapsilosis* out of a total of 11 isolates, *C. albicans* recorded strong production of 3 isolates and weak for 1 isolate, while 2 isolates of *C. glabrata* showed strong biofilm production, and 2 isolates of *C. krusei* and *C. parapsilosis* recorded strong biofilm production. Biofilm is one of the most important virulence factors for candida, which plays a major role in the infection of candidiasis. This confirms what has been achieved [22], they indicated that 60% of human infections are related to the biofilm that is formed by microorganisms that are resistant to the host's immune system and antibiotics.

The results of the current study converged with the findings [30], which indicated that *C. glabrata* gave a positive result for 48 out of 72 isolates, 63.2%, followed by *C. albicans* with 16 positive isolates from 27, 23.7%, and *C. krusei* with 3 out of 4 isolates, 3.5%. It was followed by the yeast *C. tropicales* with 6 positive isolates out of 8 isolates, with a percentage of 7%. This study is also consistent with [14] Who demonstrated the ability of both *C. tropicales* and *C. albicans* to form biofilm in a large percentage and have an ecological importance, as it helps the yeast survive as human pathogens by allowing it to escape from the host's immune mechanisms and resistance to antifungals, as well as the formation of the biofilm in *Candida* yeasts are a key factor in the survival of the species. The formation of the biofilm in *Candida* yeasts is also a key factor in the survival of the species.

[21] found everyone that 49 out of 90 isolates of *Candida* yeasts produced the biofilm, two isolates of *C. parapsilosis* made the biofilm, and *C. krusei* made the biofilm for 11 out of 18 isolates, and 3 isolates of *C. glabrata* made the biofilm out of two. 3 isolates, *C. albicans* formed the biofilm for 21 out of 41 isolates. Finally, *C. tropicales* formed the biofilm for 15 out of 26 isolates.

[30] explained Biofilm production is a characteristic of *Candida* that causes diseases, according to his study and his group, as they found that *C. albicans* and *C. glabrata* gave a high positive result for biofilm production compared to other *Candida* species.

Table (8): Biofilm formation by various *Candida* species.

<i>Candia species</i>	Total number isolation	Biofilm negative	Total Biofilm positive	strong Biofilm Positive	Weak Biofilm Positive
<i>Candida albicans</i>	4	0	4	3	1
<i>Candida glabrata</i>	4	2	2	2	0
<i>Candida tropicales</i>	1	0	1	0	1
<i>Candida krusei</i>	5	1	4	2	2
<i>Candida parapsilosis</i>	11	1	10	2	8

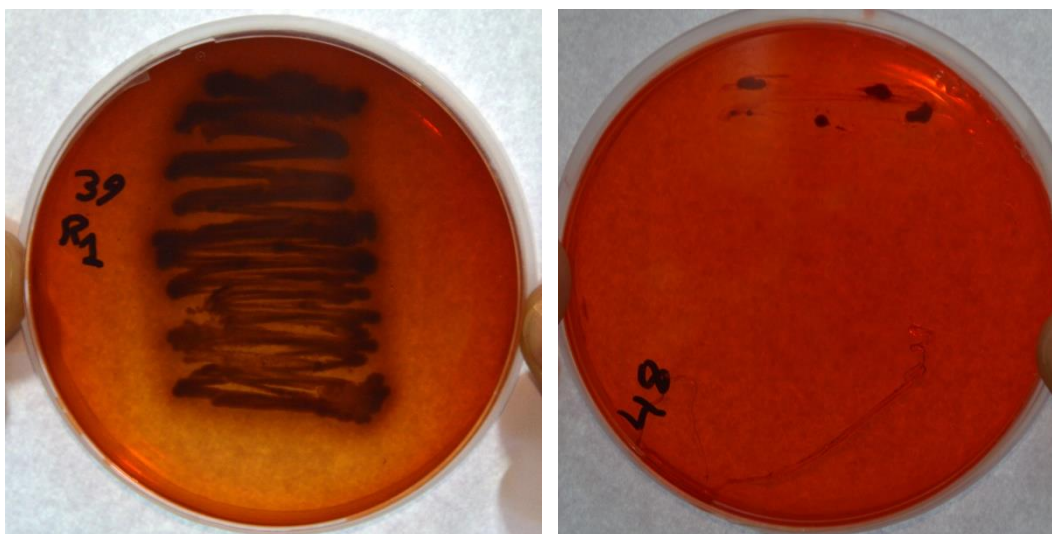


Fig. 6: Biofilm formation by *Candida* species, A: Biofilm producing; B: Non Biofilm producing.

4. Conclusion

To sum up, the results of this study showed that the most common causative agent of Otomycosis was *Candida* species particularly was *Candida parapsilosis* follow by *Aspergillus* species particularly *Aspergillus niger*. Otomycosis was most prevalent at the age of 15-40 years and the lowest

prevalence was the age 60+ years old. The sex ratio of Otomycosis was higher in female compared to for Males.

Both *C.parapsilosis* and *C.albicans* showed higher ability to form biofilm than other types of *Candida* species.

It was also noted that there is lack of research and studies related to Otomycosis that fungi as causative in Iraq in general and Mosul in particular.

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