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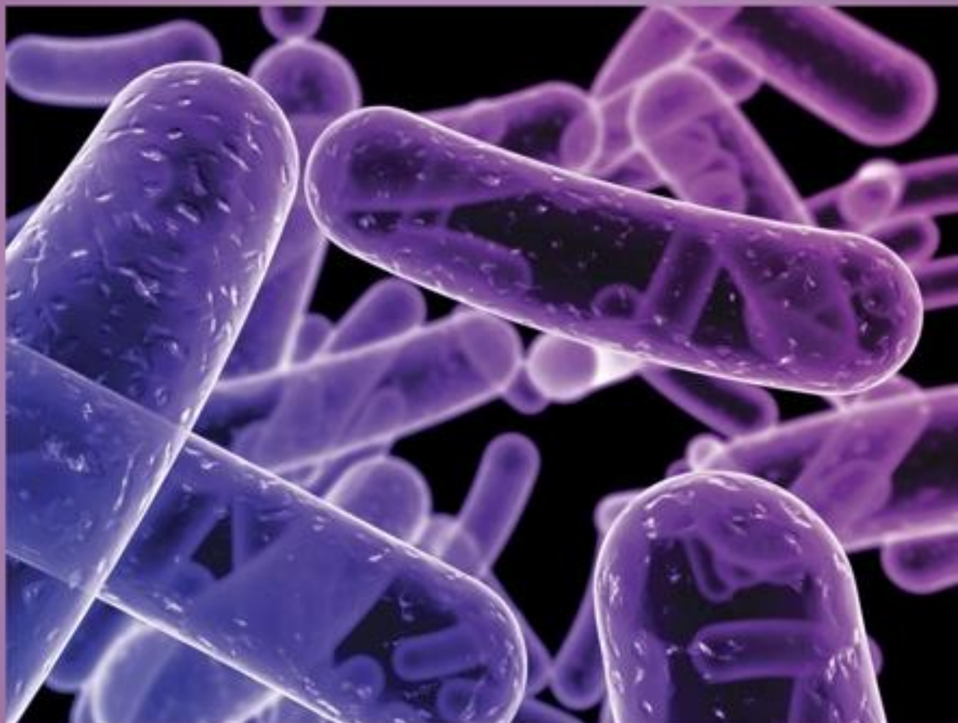

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Isolation and Diagnosis of Biofilms of *Klebsiella pneumoniae* Bacteria and *Candida albicans* Yeast, and Studying the Sensitivity of The Pathogens to Antibiotics and Antifungals

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ABSTRACT

Section describes the details of a laboratory study that was conducted between September 2022 and February 2023. The study was carried out at the laboratories of the Department of Life Sciences, College of Science, University of Baghdad, and the University of Al-Nahrain Center/Al-Nahrain University. The highest percentage of antibiotic resistance was found in *K. pneumoniae* isolates against the antibiotic Cefopime, which reached 66.67%. Meanwhile, the highest percentage of antifungal resistance was found in *C. albicans* isolates against the antifungal Metronidazole, which reached 73.33%. Yeast isolates (*C.albicans, C.glabrata, C.kefyr, C.krusei, C.parapsilosis, C.tropicalis, C.lusitaniae*) were diagnosed and tested for their ability to form biofilms on Congo red agar medium, where 40 isolates were found to be biofilm producers, with a rate of 40%.(ESBL) gene indicating an increase in the rate of antibiotic resistance in Gram-negative bacteria and their occurrence in inanimate hospital environments.

INTRODUCTION

Klebsiella pneumoniae is a notable human pathogen belonging to the Enterobacteriaceae family. Its prevalence in clinical settings has increased significantly in recent years (Vuotto *et al*., 2014), and it has now become a multidrug-resistant hospital pathogen with limited treatment options worldwide (Paczosa and Mecsas, 2016). Studies on its virulence factors have shed light on its self-protective pathogenic strategies, which primarily involve fimbriae, capsule, and lipopolysaccharide that facilitate attachment to the host surface, protection against phagocytosis, desiccation, and complement evasion, respectively (Piperaki *et al*., 2017). In addition, type 1 and type 3 fimbriae play a critical role in its colonization of passive/inert abiotic surfaces (Murphy *et al*., 2013).

Candida albicans is a dimorphic yeast that is considered an opportunistic human pathogen (Gow and Yadav, 2017). *C. albicans* is found in the flora of the human respiratory, digestive, and reproductive systems, with a prevalence of 40% to 60% in healthy adults. It is usually a commensal organism, but it can become pathogenic in individuals with immune deficiencies. It is one of the few species of the Candida genus that causes human candidiasis, resulting from an overgrowth of the fungus (Erdogan and Rao, 2015).

Biofilms consist of complex structural communities of bacteria residing within an exopolysaccharide matrix shaped by various host factors (Soto, 2013). These biofilms have been extensively studied in recent decades due to their involvement in nearly 80% of bacterial infections, particularly those associated with device-related infections, infections on body surfaces, and chronic infections (Singh *et al*., 2019). Biofilms pose a significant concern because they protect host defense mechanisms and conventional antimicrobial therapy, leading to marked impacts on the outcomes of antimicrobial treatments (Singh and Chakraborty, 2017).

The impact of microorganisms on human health is significant. However, the misuse of antimicrobial treatments and patients' failure to comply with prescribed regimens have led to the emergence of antibiotic resistance as a pressing global health concern (Karakonstantis and Kalemaki, 2019).

Resistance to the antibiotics Ciprofloxacin, Amikacin, and Piperacillin was confirmed in *K. pneumoniae* bacteria by testing them at different stages related to the formation of cell membranes, possibly because *K. pneumoniae* lung bacteria in the membrane growth pattern are more resistant to all antibiotics. The effect of Amikacin and Ciprofloxacin on small and large cell membranes was also examined at the highest achievable serum concentrations, noting that Amikacin was able to eliminate small cell membranes but became completely ineffective as membranes increased with age. A possible explanation for the increasing resistance during growth time was presented by staining the calcofluor, where improved production of external polysaccharides was observed in old cell membranes (Singla *et al*., 2013). A study by Subramanian *et al*. (2012) also showed that biofilm-producing isolates were 93.3%, 83.3%, 73.3%, and 80% resistant to nalidixic acid, ampicillin, cefotaxime, and cotrimoxazole, respectively, compared to 70%, 60%, 35%, and 60% resistance to the same antibiotics by non-biofilm-producing bacteria. Sanchez *et al*. (2013) confirmed the tendency of biofilm-forming *K. pneumoniae* strains to be more resistant to cephalosporins compared to strains that do not form biofilms.

The current study aims to isolate and diagnose the biofilms of *Klebsiella pneumoniae* bacteria and *Candida albicans* yeast, and to study the susceptibility of these pathogens to antibiotics and antifungals.

MATERIALS AND METHODS

Section describes the details of a laboratory study that was conducted between September 2022 and February 2023. The study was carried out at the laboratories of the Department of Life Sciences, College of Science, University of Baghdad, and the University of Al-Nahrain Center/Al-Nahrain University.

Bacterial samples were collected from the respiratory system and diagnosed using the Vitek2 Compact system. The *Klebsiella* spp isolates were obtained from educational laboratories in Al-Tib City/Baghdad.

Yeast samples were collected from Al-Tib Hospital and Yarmouk Hospital in Baghdad province, from individuals with respiratory diseases. The yeast isolates included *C. albicans, C. glabrata, C. kefyr, C. krusei, C. parapsilosis, C. rangosa, and C. tropicalis*, and were identified using the Vitek System.

Isolation and Identification of Microbial Isolates: The identification of microbial isolates was carried out by performing various biochemical tests based on the protocol

developed by Ellen *et al*. (1994). The following steps were carried out:

Cultivation of Isolates: The isolates obtained from solid Sabouraud Dextrose Agar (SDA) were cultivated and incubated at 37°C for 2-4 days. To reactivate the isolates, three replicates were made for each isolate, and the surface colonies were examined for their shape, color, diameter, and height (Ellis *et al*., 2007).

Staining of Isolates: A small portion of the growing colony on corn-meal agar was taken using a loop and mixed with lactophenol blue stain on a glass slide. The slide was covered with a cover slip and observed for the presence of chlamydospores and yeast cells.

Growth on Chromo Agar Medium: The isolates were incubated on SDA at 37°C for 48 hours before being transferred to Chromo agar medium. The medium was then incubated for 48-72 hours at 37°C. Chromo agar is a selective medium for identifying Candida isolates such as *C. krusei, C. albicans, C. glabrata, C. sphaerica, C. parapsilosis, C. kefyer*, *C.incospicua*, based on the shape and color of the colonies produced by the isolate (Hospenthal *et al*, 2006). The strains were identified according to the manufacturer's instructions, which determine the Candida species based on the color and external appearance of the colony.

Diagnosis of Pathogens: Diagnosis of bacterial isolates: The bacterial isolates under study were diagnosed by performing a microscopic examination and determining their microscopical and cultural characteristics after growing them on specific culture media for initial isolation. They were initially diagnosed based on their morphological and cultural characteristics, including colony size, color, edges, and elevation. Then, their characteristics were studied under the microscope after Gram staining (Forbes *et al*., 2007).

Diagnosis of Yeast Isolates: This test was performed using the Vitek2 Compact System based on the manufacturer's instructions and as described in a study by Kaur *et al*. (2016).

Biochemical Test:

- **Chlamydospores Test:** This test is performed to observe chlamydospores in the false hyphae ends according to the method described by Ferreira *et al*. (2010).
- **Germ Tube Forming Test:** This test is performed to observe the distinctive germ tube formation for *C. albicans* and *C. dubliniensis* as described by Ellis *et al*. (2007).
- **Test for Pathogens' Ability to Form Biofilms:**

  Test the Ability of Candida to Produce Biofilms: This was done by testing on Congo Red Agar (CRA) medium according to Oliveira and Cunha's method (2010).

  Test the Ability of Klebsiella Bacteria to Form Biofilms: The ability of Klebsiella spp. isolates to form biofilms were detected using the Microtiter plate method as described by Adriana *et al* (2013).
- **Antimicrobial Susceptibility Test:**

  Antibiotic Susceptibility Test: The sensitivity of 90 clinical isolates of Klebsiella spp. to nine antibiotics was tested using the disc diffusion method according to Bauer *et al*. (1966).

  Antifungal Susceptibility Test: The sensitivity test for yeast was performed on the Muller-Hinton agar medium by transferring colonies with a sterile cotton swab onto the surface of the medium and leaving them for 15 minutes to dry. Then, antibiotic discs were transferred to the surface of the agar using forceps and incubated at 37°C for 24-48 hours. The results were read using a ruler by observing the inhibition zone diameters around the antifungal discs (AL-Bajilan, 2016).

RESULTS AND DISCUSSION

1-Morphological and Microscopic Characteristics for Diagnosis of Yeast Isolates:

The diagnosis of yeast isolates (*C. albicans, C. glabrata, C. kefyr, C. krusei, C. parapsilosis, C. tropicalis, C. lusitaniae*) was

confirmed by the Vitek system according to the method of Cletus and Jack (1998). The isolates were identified based on their morphological and other cultural and biochemical characteristics. Members of this genus appeared as smooth, creamy-white to milk-white colonies when grown on Sabouraud Dextrose Agar chloramphenicol (SDAC) medium for 2-3 days at 37°C. The colonies were examined microscopically after staining with lactophenol blue and it was observed that the cells appeared spherical to oval or elongated and budding. Pseudohyphae were also occasionally present, which is consistent with Bhavan *et al*. (2010).

2- Microscopic Characteristics:

When stained with cotton-blue lactophenol stain, *C. albicans,* and *C. tropicalis* appeared as spherical to sub-spherical budding cells, while C. Lusitania appeared as oval to fusiform budding cells. C. parapsilosis appeared as small spherical to oval budding cells, and C. krusei appeared as elongated to oval budding cells. C. kefyr appeared as short oval budding cells, which is consistent with the findings of Boon *et al*. (2013). The cells were also stained with Gram stain (crystal violet) as shown in Figure (1) and gave positive results where they were colored purple. This is due to the presence of a thick layer of peptidoglycan in their cell wall (Sudbery *et al*., 2004).

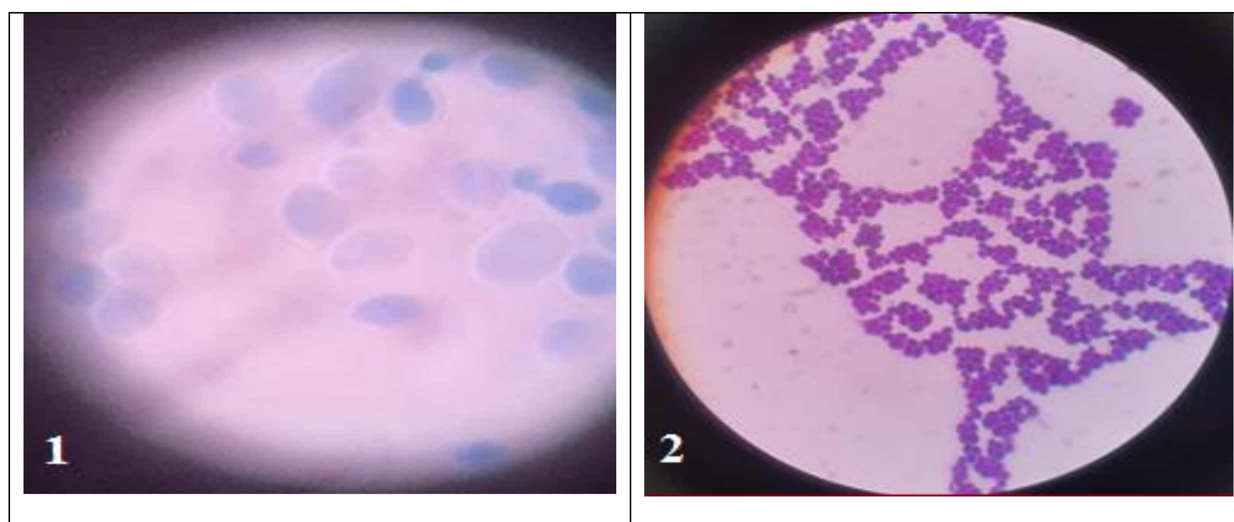

Fig.1: Yeast *C. albicans*, 1 (stained with cotton-blue lactophenol stain, 2) stained with Gram stain (crystal violet) (100X).

3- Diagnosis of Yeasts using Corn Meal Agar (CMA) Medium:

This medium is used to detect the ability of these yeasts to form chlamydospores (Fig.2). The results obtained (Table 1) showed that all studied yeasts were unable to form chlamydospores when grown on this medium except for *C. albicans*, which was consistent with the results of (Saroj *et al*., 2013; Al-Saadi, 2015). The colors were also consistent with the manufacturer's description of the medium, where circular, thick-walled chlamydospores were formed at the ends of the fungal threads, either singly or in clusters when these yeasts were cultured on CMA medium. The reason for the formation of these chlamydospores is due to the starvation of these yeasts and a lack of nutritional sources since these spores are formed when conditions are unfavorable. This medium is described as a starvation medium for yeasts (Saroj *et al*., 2013).

Table 1: Shows the ability of yeasts to form chlamydospores in a CMA medium.

Candida spp.	***C.albicans***	***C.glabrata***	***C.krusei***	***C.kefyr***	***C.parapsilosis***	***C.tropicalis***	***C.lusitaniae***
Result	+	-	-	-	-	-	-

+ Indicates yeast's ability to form chlamydospores.
- Indicates yeast's inability to form chlamydospores.

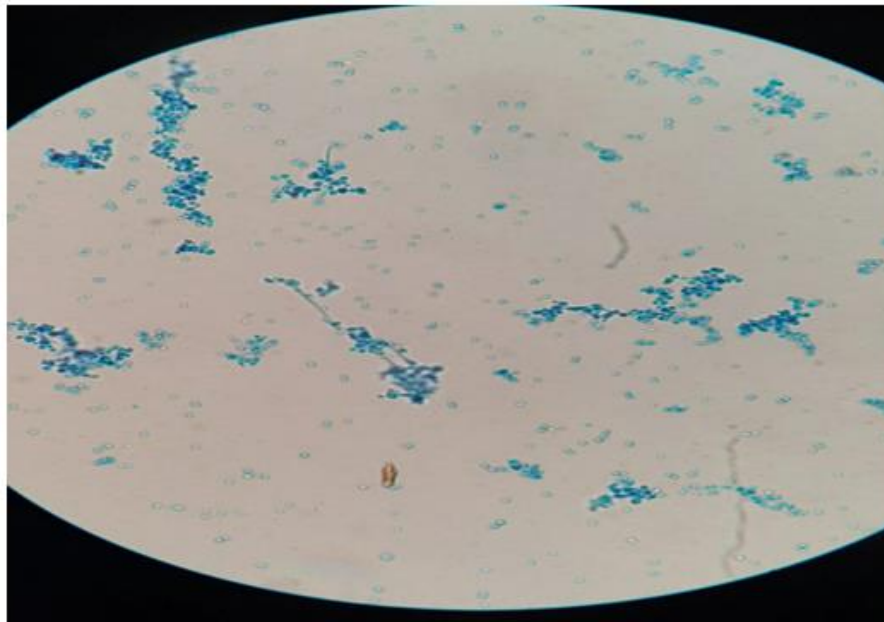

Fig. 2: Formation of chlamydospores by *C. albicans* after 24 hours of incubation at 25°C with a magnification of 40x.

4-Diagnosis of yeast using Chrom Agar Culture Medium:

The growth test on Chrom Agar culture medium is one of the most effective and rapid biochemical tests in diagnosing different types of yeasts based on their color after inoculation and incubation compared to other tests. The results of the test showed colonies of different colors on the Chrom Agar medium. *C. albicans* colonies appeared light green, while *C. parapsilosis* yeast appeared white and *C. glabrata* colonies appeared pink. The remaining isolates appeared in various shades of red and brown, as shown in Figure (3) and Table (2). These results are consistent with those reported by David and Stephen (2007), Nadeem *et al.* (2007), and Kumar and Edward (2014).

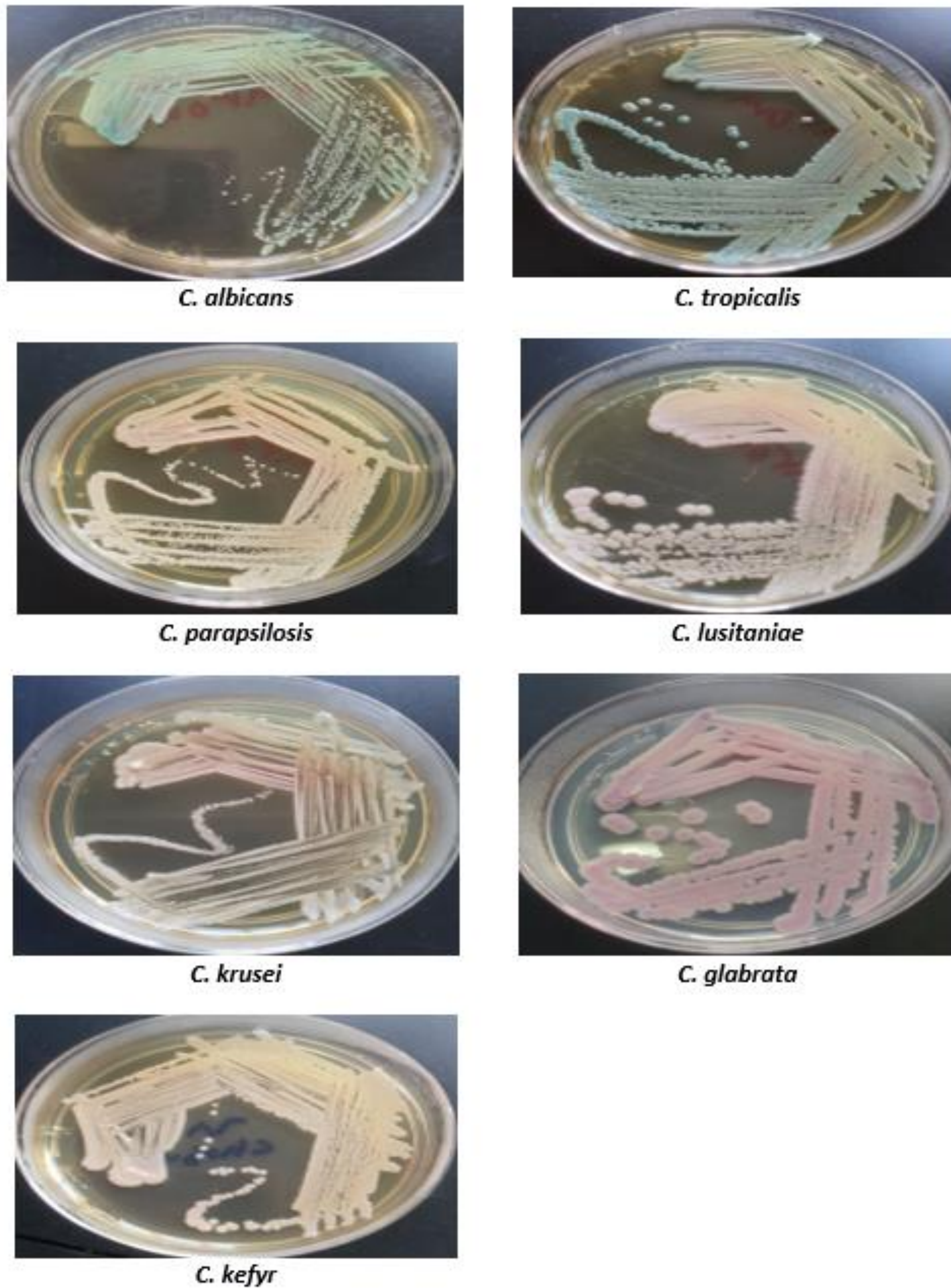

Fig. 3: Colors of *Candida* yeast isolates on HiCrome Differential Agar.

Table 2: Candida spp. isolated according to color on HiCrome Candida Differential Agar.

***Candida* spp.**	**The color on HiCrome Candida Differential Agar**
C. albicans	Light green
C. parapsilosis	White to creamy
C. krusei	Pale purple
C. kefyr	Creamy to white
C. tropicalis	Greenish-blue
C. glabrta	Creamy to pink
C. lusitania	Creamy to pink

5- Germ tube formation test: The results of the germ tube formation test (Table 3) showed that all *C. albicans* isolates were able to form germ tubes at a rate of 100%, as shown in Figure (4), while the other species did not form germ tubes. These results were consistent with those reported by Kumar and Shukla (2010) and Al- Hamadani (2020).

The ability to form germ tubes is an important virulence factor closely related to the pathogenicity of *C. albicans*. The importance of germ tubes lies in enabling the yeast to adhere to the host's epithelial cell surfaces for infection. Germ tube formation is observed when *C. albicans* transforms from yeast form to hyphal form (Yang, 2003, Abdulbaqi *et al*.,2018).

Table 3: The ability of Candida spp. isolates to form germ tubes.

Candida spp.	*C. albicans*	*C. glabrata*	*C. krusei*	*C. kefyr*	*C. parapsilosis*	*C. tropicalis*	*C. lusitaniae*
Germ tube	+	-	-	-	-	-	-

+ Indicates yeast's ability to form a Germ tube.

- Indicates yeast's inability to form a Germ tube.

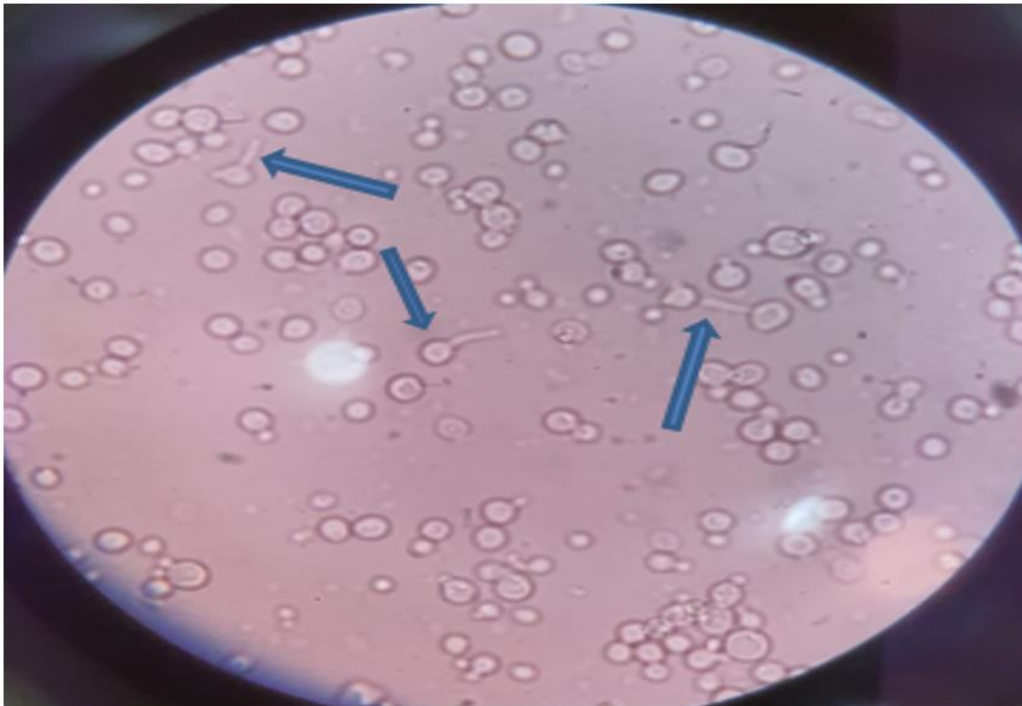

Fig. 4: Formation of germ tube of *Candida albicans* yeast cell grown in human blood serum after two hours of incubation at 37°C (40x).

6: Types of Candida Species Isolated and Identified During the Study:

Table (4) shows the numbers and percentages of isolated *Candida* species during the study after conducting all the tests presented earlier. The number of *C. albicans* isolates was 64, accounting for 64% of the total isolates, compared to non-albicans *Candida* isolates, which accounted for 36% and were distributed among the tested species as follows: 9 isolates of *C. tropicalis* (9%), 3 isolates of *C. Lusitaniae* (3%), 8 isolates of *C. parapsilosis* (8%), 6 isolates of both *C. glabrata* and *C. krusei* (6% each), and 4 isolates of *C. kyfer* (4%). These results are consistent with those reported by Arora *et al*. (2017), who found that the percentage of *C. albicans* was 63.4%, and the other non-

albicans *Candida* species accounted for 36.6%. The results are also consistent with those reported by Habib *et al*. (2015), who isolated four species, with *C. albicans* being the most common, followed by *C. tropicalis, C. parapsilosis*, and *C. krusei*. These findings are in agreement with De Sousa *et al*. (2016) and Thiyahuddin *et al*. (2019), who focused on isolating *Candida* from the oral cavity, with *C. albicans* being the most frequently isolated species. The results are not consistent with those reported by Al-Tekreeti *et al*. (2017), who found that only 6 out of 30 isolates were *C. albicans*, or with Vainionpää *et al*. (2019), who did not report any *C. albicans* infection and only isolated *C. parapsilosis*.

The increase in the incidence of candidiasis can be attributed to the frequent use of antibiotics, poor dietary habits of the infected person, and the host's immunity, all of which increase the risk of infection.

Table 4: Shows the numbers and percentages of isolated *Candida* spp. species.

***Candida* spp.**	**Numbers**	**Percentages**
C. albicans	64	64%
C. tropicalis	9	9%
C. lusitaniae	3	3%
C. parapsilosis	8	8%
C. glabrata	6	%6
C. kefyr	4	4%
C. krusei	6	6%
Total	100	100%

The reason for the increase of *C. albicans* yeast over other types of Candida can be attributed to its ability to adhere strongly to host surfaces, causing candidiasis, and its ability to form large colonies and inhibit host defense mechanisms in various parts of the body (Dhanasakaran *et al*., 2014), in addition to other virulence factors such as its ability to form hyphae, undergo a morphological transformation and form the biofilm (Rouabhia and Chmielewski, 2012, Dheeb *et al*.,2015). Furthermore, it can secrete enzymes such as Aspartic Proteinase responsible for protein breakdown, and Phospholipase responsible for breaking down phospholipid fats, which are the main component of the yeast cell membrane (Dantas *et al*., 2016). Although it is a natural flora in the body, it can become pathogenic under suitable conditions and weaken host immunity. Moreover, the overall increase in fungal infections is attributed to the use of medical devices (catheters), parenteral nutrition, and the indiscriminate use of antibiotics (Sharma *et al*., 2017, Dheeb *et al*.,2014).

7- Distribution of candidiasis infections by gender: Figure (5) demonstrates significant differences in the infection rate between males and females at a probability level of ($P \leq 0.01$), and that the majority of candidiasis infections are attributed to females, with 68 isolates and a percentage of 68%, compared to 32 isolates and a percentage of 32% for males. These results are consistent with a study by Cataldi *et al*. (2017), who isolated candida from different clinical sites and recorded a higher infection rate in females at 62% compared to males at 38%. A similar trend was observed by Abdulla and Mustafa (2020), with a female infection rate of 56% compared to males at 44%, while it did not agree with the findings of Al-Hamadani (2020), who reported a male infection rate of 58.44% and a female infection rate of 41.46%. The results also did not align with the study by Arastehfar *et al*. (2018), which reported an infection rate of 62.4% in males and 37.6% in females.

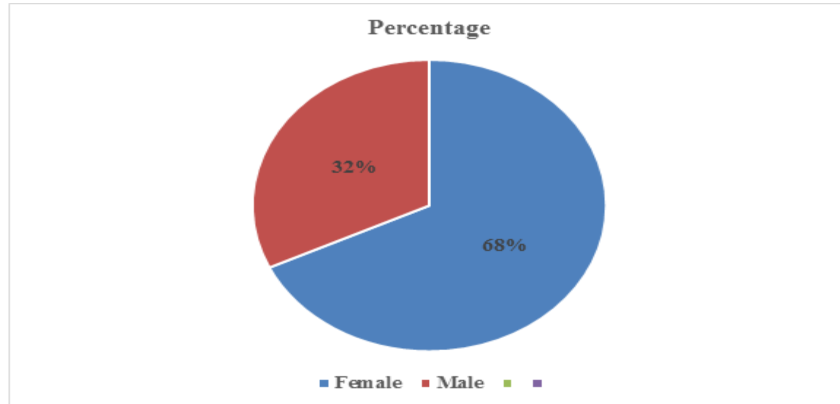

Fig. 5: Illustrates the percentage of infection based on gender.

8- Biofilm Detection Tests:

The ability of isolates to form biofilms on Congo red agar medium was tested, where 40 isolates produced biofilms with a percentage of 40%. This percentage is similar to what Saxena *et al*. (2014) found, where their isolates produced biofilms on Congo red agar at a percentage of 41.8%. However, it did not match what Khalaf (2016) found, where the percentage of their isolates that produced biofilms was 51.6%.

These results were obtained through the colony morphology on Congo red agar medium, where the colonies of biofilm-producing isolates appear as dry or shiny black, while the colonies of non-biofilm-producing isolates appear as light pink. The color change in the colonies in this way, which occurs in the late stages of the incubation period, may be due to the presence of secondary metabolites. The use of 5% sucrose or glucose is described as a basic factor in determining the production of extracellular polysaccharides using nutrient-rich media (Oliveira and Cunha, 2010). The color change may be due to the direct binding of Congo red dye to certain polysaccharides that make up specific complexes (Hassan *et al*., 2011, Nouri *et al*.,2015).

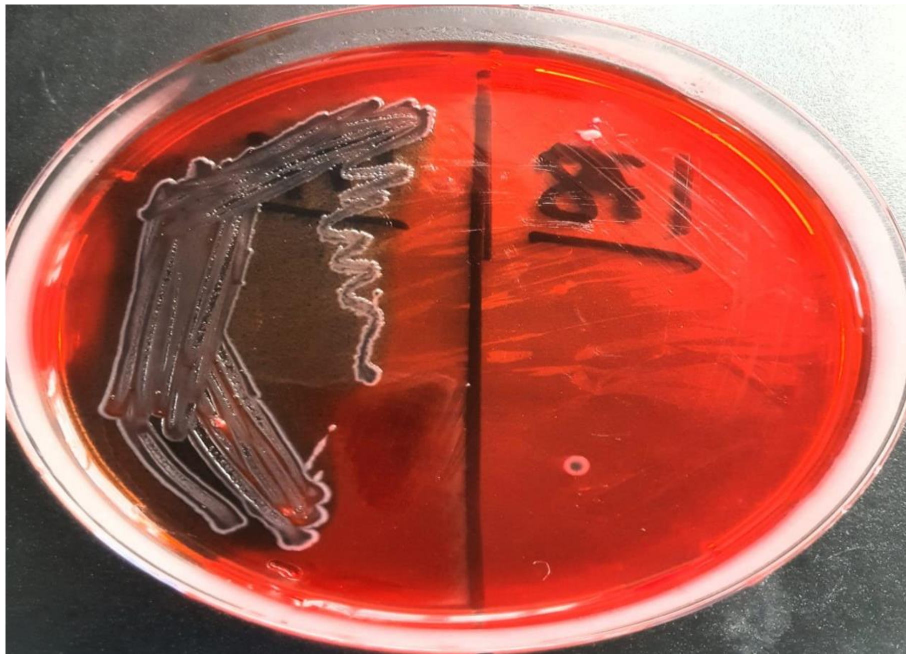

Fig.6: Shows the production of *C. albicans* biofilm on the surface of a red Congo medium.

9- Sensitivity testing of *K. pneumoniae* and *C. albicans* to some antibiotics and antifungal agents: Results from Table (5) indicate the sensitivity of the studied isolates of *K. pneumoniae* and *C. albicans* to some antibiotics and antifungal agents, with a total of 21 and 45 isolates studied for each type, respectively. Regarding the *K. pneumoniae* isolates, it is evident that 33.33% of them were resistant to Gentamicin, 52.38% were sensitive, and 14.29% had moderate sensitivity. For Ceftriaxone, 42.86% were resistant, 33.33% were sensitive, and 23.81% had moderate sensitivity. Additionally, 23.81% of the isolates were found to be resistant to Amikacin, while 61.9% were sensitive and 14.29% had moderate sensitivity. For Amoxyclav, 38.1% were resistant, 47.62% were sensitive, and 14.29% had moderate sensitivity. As for Cefopime, 66.67% of the isolates were resistant, 19.1% had moderate sensitivity, and 14.29% were sensitive. In terms of Ciprofloxacin, 38.1% were found to be resistant, 4.76% had moderate sensitivity, and 57.14% were sensitive. The percentage of isolates resistant to Meropenem was 19.1%, while the percentage of sensitive isolates was 80.9%. The percentage of isolates resistant to Tobramycin was 28.57%, while the percentage of sensitive isolates was 57.14%. Additionally, 14.29% of the isolates had moderate sensitivity.

Regarding *C. albicans* isolates, it was found that 42.22% of them were resistant to the antifungal Voriconazole, while 44.44% were sensitive and 13.33% showed intermediate sensitivity. For Miconazole, 35.56% were resistant, 48.89% were sensitive, and 15.56% showed intermediate sensitivity. Meanwhile, 28.89% of the isolates showed resistance to Ketoconzole, 66.67% were sensitive, and 4.44% showed intermediate sensitivity. All studied isolates showed varying levels of resistance to the antifungal Metronidazole, with 73.33% being resistant and 26.76% showing intermediate sensitivity. For Nystatin, 2.22% of the isolates were resistant, 13.33% showed intermediate sensitivity, and 84.44% were sensitive. As for Fluconazole, 46.67% of the isolates were resistant, 11.11% showed intermediate sensitivity, and 42.22% were sensitive. The percentage of isolates resistant to Traconazole was 13.33%, while 75.56% were sensitive and 11.11% showed intermediate sensitivity. The percentage of isolates that are resistant to the antifungal Clotrimazole was 4.44%, while 82.22% were sensitive and 13.33% showed intermediate sensitivity. Candida isolates showed varying sensitivity to the antifungal Amphotericin, with 91.11% of the isolates being sensitive and 8.89% showing intermediate sensitivity.

As mentioned above, the highest percentage of antibiotic resistance in *K. pneumoniae* isolates was recorded against the antibiotic Cefopime, while the highest percentage of antifungal resistance was found in *C. albicans* isolates against the antifungal Metronidazole.

Table 5: Sensitivity of *K. pneumoniae* and *C. albicans* isolates to some antibiotics.

Antibiotics	*K. pneumoniae* (n=21)			Antifungals	*C. albicans) n*=45)		
	R	M	S		R	M	S
Gentamicin	7(33.33)	3(14.29)	11(52.38)	Voriconazole	19(42.22)	6(13.33)	20(44.44)
Ceftriaxone	9(42.86)	5(23.81)	7(33.33)	Miconazole	16(35.56)	7(15.56)	22(48.89)
Amikacin	5(23.81)	3(14.29)	13(61.9)	Ketoconzole	13(28.89)	2(4.44)	30(66.67)
Amoxyclav	8(38.1)	3(14.29)	10(47.62)	Metronidazole	33(73.33)	12(26.67)	0
Cefopime	14(66.67)	4(19.1)	3(14.29)	Nystatin	1(2.22)	6(13.33)	38(84.44)
Ciprofloxacin	8(38.1)	1(4.76)	12(57.14)	Fluconazole	21(46.67)	5(11.11)	19(42.22)
Meropenem	4(19.1)	0	17(80.9)	Traconazole	6(13.33)	5(11.11)	34(75.56)
Tobramaycin	6(28.57)	3(14.29)	12(57.14)	Clotrimazole	2(4.44)	6(13.33)	37(82.22)
-	-	-	-	Amphotericin	0	4(8.89)	41(91.11)

Resistance (R), intermediate sensitivity (M), and sensitivity (S).

Long-term prophylactic use of antibiotics and antifungals in suspected cases is a contributing factor to the high resistance pattern to these drugs. Roy *et al.* (2013) confirmed that the proper use of antifungal agents in hospitals reduces drug resistance. Another proven fact is that the response of antifungal drugs in the laboratory may depend on the dose and is expressed as the sensitive dose (SDD). In other words, although it is sensitive in the laboratory, resistance failure can be seen in the living body at the usual dose. In such cases, increasing the dose of the drug above the usual dose often leads to clinical treatment (Saha *et al*., 2008). Adhikary R, Joshi (2011) showed that fungal species are highly susceptible to antifungal agents from the polyenes, azole, and echinocandin categories, which showed very high resistance when exposed to fluconazole for all isolates despite being highly sensitive to other antibiotics such as Amphotericin. This is consistent with the results of our study, which showed that fungal isolates have high to moderate resistance to azole antifungals compared to other antibiotics such as Amphotericin and Nystatin.

High resistance in biofilm-forming pathogens is not solely because these biological membranes are highly tolerant to antimicrobials and host defense mechanisms, but also because of their mechanisms of accommodation under various physiological conditions, such as growing and adapting to stress and dormancy. For instance, Staphylococcus epidermidis bacteria protect themselves against the human fungal immune system by using EPS adhesion between cells (Brescó *et al*., 2017). Furthermore, bacteria that produce biofilms have a higher rate of horizontal gene transfer compared to planktonic cells, and the mutation rate is higher in biological membranes than in planktonic cells (Borges *et al*., 2016, EL-Hilali *et al.,* 2016). In addition, biological membranes increase the likelihood of gene transfer with the aid of virulence factors and antibiotic-resistance genes from antibiotic-resistant bacterial species, resulting in antibiotic resistance (Galloway *et al*., 2012). When patients are infected with biofilm-forming pathogens, it becomes difficult to treat them even with high doses of antibiotics, which can lead to their death (Abraham, 2016). For example, treating and removing disease-causing biological membranes from patients requires doses of antibiotics that are 10-1000 times higher than the equivalent strain that exists in planktonic form, as integrated bacterial cells acquire an ideal defense mechanism against the harmful effects of antibiotics and the host's immune system (Hughes and Webber, 2017).

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