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ORIGINAL ARTICLE

# Identification and in Vitro Susceptibility Pattern of Fungal Pathogens in Immunocompromised Patients with invasive Fungal Infections

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***Key words:***
***Invasive fungal infections, Immunocompromised, Brilliance Candida agar, Candida ,aspergillus, Antifungal agents***

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## ABSTRACT

***Background****: In intensive care units, invasive fungal infections have become more common, particularly among immunocompromised patients. Early identification and starting the treatment of those patients with antifungal therapy is critical for preventing unnecessary use of toxic antifungal agents.* ***Objective:*** *The aim of this research is to determine which common fungi cause invasive fungal infection in immunocompromised patients, as well as their antifungal susceptibility patterns in vitro, in Assiut University Hospitals.* ***Methodology:*** *This was a hospital based descriptive study conducted on 120 patients with clinical suspicion of having fungal infections admitted at different Intensive Care Units (ICUs) at Assiut University Hospitals. Direct microscopic examination and inoculation on Sabouraud Dextrose Agar (SDA) were performed on the collected specimens. Isolated yeasts were classified using phenotypic methods such as chromogenic media (Brilliance Candida agar), germ tube examination, and the Vitek 2 system for certain isolates, while the identification of mould isolates was primarily based on macroscopic and microscopic characteristics. Moulds were tested in vitro for antifungal susceptibility using the disc diffusion, and yeast were tested using Vitek 2 device cards.* ***Results:*** *In this study, 100 out of 120 (83.3%) of the samples were positive for fungal infection. Candida and Aspergillus species were the most commonly isolated fungal pathogens. The isolates had the highest sensitivity to Amphotericin B (95 %), followed by Micafungin (94 %) in an in vitro sensitivity survey.* ***Conclusion:*** *Invasive fungal infections are a leading cause of morbidity and mortality in immunocompromised patients, with Candida albicans being the most frequently isolated yeast from various clinical specimens; however, the rise in resistance, especially to azoles, is a major concern.*

## INTRODUCTION

Fungal infections are becoming more prevalent at an unprecedented pace, creating a major diagnostic and treatment challenge for healthcare professionals. An increase in the number of immunocompromised patients admitted to intensive care units may be one explanation for the rise in fungal infections. Patients with granulocytopenia, HIV infection, bone marrow and solid organ transplantation, cancer, diabetes mellitus, and other predisposing factors for poor immunity are at an elevated risk.[1]

*Candida* species and *Aspergillus* species are the most common fungal pathogens isolated from immunocompromised patients. *Cryptococcus* species, *Zygomycetes*, and *Mucormycetes*, are the other most important aetiologic agents.[2]

Fungal infections can range from minor to serious. Invasive fungal infections (IFI) were less common, but they can damage any organ in patients who are critically ill.[۲] and are linked to high rates of morbidity and mortality .[3]

The lack of distinct signs of infection and the poor sensitivity of traditional culture-based approaches for diagnosing fungal diseases have been blamed for the high morbidity of invasive fungal infections. This has resulted in delayed antifungal treatment, which has a detrimental effect on outcomes. As a result, early and reliable diagnosis is critical for prompt antifungal therapy implementation, reducing antifungal agent side effects and the use of empirical antifungal therapy, and thereby reducing the emergence of antifungal resistance.[4] Prophylaxis, which has been shown to be beneficial in clinical environments, is the first strategic choice.[5]

Antifungal susceptibility testing is conducted to help decide which medication is best for treating a serious fungal infection. Antifungal susceptibility testing for isolates has become important due to changing

epidemiology of fungal agents, increasing drug resistance, availability of more and newer antifungals, and increasing practise of prophylactic use. [6]

Invasive fungal infections have a limited treatment options.The first drug to be introduced was amphotericin B (polyene), which was followed by flucytosine (pyrimidine) and the first generations of azoles (fluconazole, itraconazole). Echinocandins (caspofungin, micafungin, anidulafungin) and triazoles (voriconazole, posaconalzole) have recently become available. Although most fungi are still susceptible to many of these drugs, increased use of antifungal therapy has resulted in higher resistance rates among clinical isolates, possibly due to a rise in the number of immunocompromised people and the use of azoles.[7]

The aim of our research is to determine which common fungi cause invasive fungal infection in immunocompromised patients, as well as their in vitro antifungal susceptibility patterns.

## METHODOLOGY

**Study design and population:**

From May 2019 to May 2020, a laboratory-based descriptive research was performed in the Microbiology Unit of the Clinical Pathology Department at Assiut University Hospital. The research included 120 patients who were admitted to various intensive care units (ICUs) at Assiut University Hospitals, including the Chest ICU, critical ICU, Internal Medicine ICU, Trauma ICU, and Haematology ICU.

Sputum samples (n=57), blood samples (n=24), and urine samples (n=39) were taken from the patients.

Approval number of ethical committee: 17100349

**Laboratory processing of samples:**

*Direct microscopic examination:*

Lactophenol Cotton Blue (LCB) and Gram stain were used to prepare direct smears from samples and test them.

*Culturing of samples:*

Two Sabouraud dextrose agar plates (HiMedia, India) were used to culture the samples, which were supplemented with chloramphenicol (0.5 mg/l). For at least 2 weeks, one was incubated at 37°C and the other at 25°C, with regular examinations until fungal colonies appeared or were confirmed as sterile and negative. For further studies, the developing fungi were held in SDA slants and sterile eppendorf tubes containing sterile glycerol (15%) in distilled water (85%).

*Identification of isolated fungi:*

- *Yeast identification methods:-*
  - Culture on chromogenic media: Brilliance Candida differential agar (Oxoid Company, UK), as stated in the included pamphlet, to provide identification of yeast isolates based on colony colour.
  - Germ tube test: This is a simple way to distinguish *Candida albicans* from other *Candida* species. Reynolds – Braude Phenomenon is another name for this phenomenon. [8]
  - Inoculation of yeast isolates on cornmeal agar (HiMedia, India): chlamydospore production identifies *Candida* species.[9]
  - Vitek 2 system cards:it is rapid and accurate method .
- *Techniques for identifying filamentous fungi:*
  - Macroscopic morphology on SDA: After obtaining sufficient growth on SDA, morphologic features are used to distinguish filamentous fungi.
  - Microscopy: LCB stained wet mount showed distinctive fungal morphology.[10]

**Antifungal susceptibility testing of isolated fungi:**

Susceptibility testing of isolated fungi was carried out using the following methods:

*The disc diffusion method :-* It's used to test mould isolates using the CLSI M51-A mould disc diffusion process.[11] Polyenes (Amphotericin B 100 units), azoles (Fluconazole 25 g, Voriconazole 1 g), echinocandins (caspofungin,micafungin), and antimetabolites (5-floucytosin ) were all tested as antifungal agents

*Vitek 2 system cards:-* It is automated antifungal susceptibility test for yeast

## RESULTS

Direct Microscopic Examination (DME) and specimen culture: Direct microscopic examination revealed that 21 (17.5%) of the 120 samples tested positive for fungal infection, while 100 (83.3%) tested positive for fungal growth when inoculated on Sabouraud Dextrose Agar (SDA). Out of the 100 positive samples, 90 (90%) revealed yeast fungal infection, while the remaining 10 (10%) had filamentous fungi as a source of infection (table 1).

**Table 1: Culture results of the collected samples.**

| Culture result | N= 100 |
|---|---|
| Yeast | 90 (90%) |
| Mold | 10 (10%) |

**Identification of isolated fungi**:

*Identification of yeast isolates:* A total of 90 yeast isolates were extracted from the 100 positive cultures, with 87 yeast isolates (96.7%) being detected phenotypically on Brilliance Candida agar (Fig.1) and by Germ tube examination, and three isolates (3.3%) being phenotypically unidentifiable.

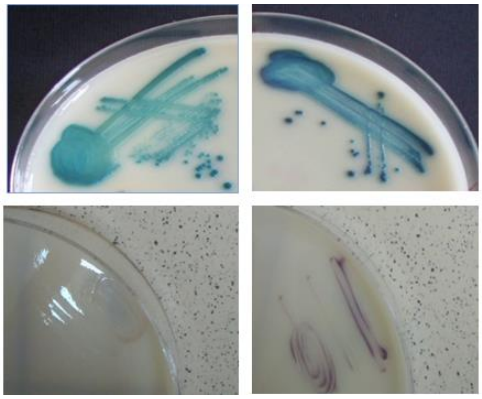

**Fig. 1:** Candia isolates colony colour on Brilliance Candida agar

**Table 2: After presumptive recognition based on phenotypic characteristics, the total number and percentage of yeast isolates are determined.**

| Presumptive Identification | N= 90 | Brilliance candida Agar | Germ tube test |
|---|---|---|---|
| ***C.albicans*** | 35 (38.9%) | Green | **34 + ve,1 –ve** |
| ***C.tropicals*** | 34 (37.8%) | Dark blue | **–ve** |
| ***C.krusei*** | 10 (11.1%) | Pink brown | **–ve** |
| ***C.glabrata*** | 8 (8.9%) | Beige | **–ve** |
| **Unknown** | 3 (2.7%) | no growth | **–ve** |

87 isolates were defined using traditional methods. The vitek 2 systems classified the three unidentified isolates as two *C.famata* strains and one *C. parapsilosis* strain.

The percentages of yeast strains were: *C. albicans* 38.9%, *C. tropicalis* 37.8%, *krusei* 11.1%, *C. glabrata* 8.9%, *C.famata* 2.2% and *C. parapsilosis* 1.1%, the result is shown in table 2 and Fig. 2.

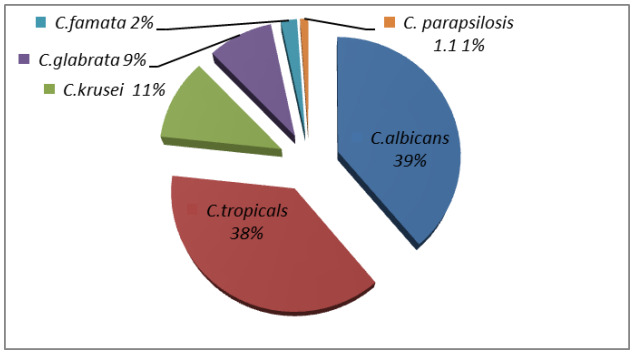


**Fig. 2:** Percentage of yeast isolates in the study

*Identification of isolated filamentous fungi:* Ten mould isolates were isolated in samples from 100 positive cultures, phenotypically classified as *Aspergillus niger* 4 (4%), *Aspergillus flavus* 4 (4%), and *Aspergillus penicillium* 2 (2%) (Fig. 3).

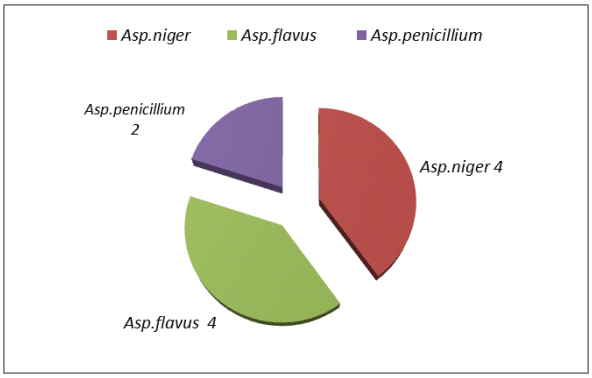


**Fig. 3:** Percentage of mould isolates in the study

*Type of isolates based on different risk factors:*

Antibiotic use was the most common risk factor (75 %) among the patients in this study, followed by diabetes mellitus (25%). *C. albicans* and *C. tropicalis* were the most common isolates in patients with these risk factors (table 3).

**Table 3: Types of isolates based on different risk factors**

| | **Antibiotic use (n= 75)** | **DM (n= 25)** | **Steroid therapy (n= 18)** | **Mechanical ventilation (n= 13)** | **Hematological malignancies (n= 12)** | **Non-hematological malignancy (n= 10)** |
|---|---|---|---|---|---|---|
| *C.tropicals* | 25 (33.3%) | 9(36%) | 9 (50%) | 7 (53.8%) | 0 | 5 (50%) |
| *C.albicans* | 23 (30.7%) | 9(36%) | 5 (27.8%) | 3 (23.1%) | 8 (61.5%) | 3 (30%) |
| *C.krusei* | 10 (13.3%) | 2 (8%) | 2(11.1%) | 1 (7.7%) | 2 (15.4%) | 0 |
| *C.glabrata* | 7 (9.3%) | 2 (8%) | 1 (5.6%) | 0 | 1 (7.7%) | 2 (20%) |
| *C.famata* | 1 (1.3%) | 1 (4%) | 0 | 0 | 0 | 0 |
| *C.parapsilosis* | 1 (1.3%) | 0 | 0 | 0 | 0 | 0 |
| *Asp.penicillium* | 2 (2.7%) | 0 | 0 | 0 | 0 | 0 |
| *Asp.niger* | 4 (5.3%) | 0 | 1 (5.6%) | 2 (15.4%) | 1 (7.7%) | 0 |
| *Asp.flavus* | 2 (2.7%) | 2 (8%) | 0 | 0 | 0 | 0 |

**Antifungal Susceptibility Testing (AST):**

The antifungal susceptibility of 100 fungal isolates was tested using six different antifungal agents, as shown in Fig. (4). The most susceptible antifungal drugs were Amphotericine B, Micafungin, and Caspofungin, which affected 95 %, 94%, and 86 % of the strains, respectively, and the most resistant drugs were fluconazole (35 %) and flucytosine (29%).

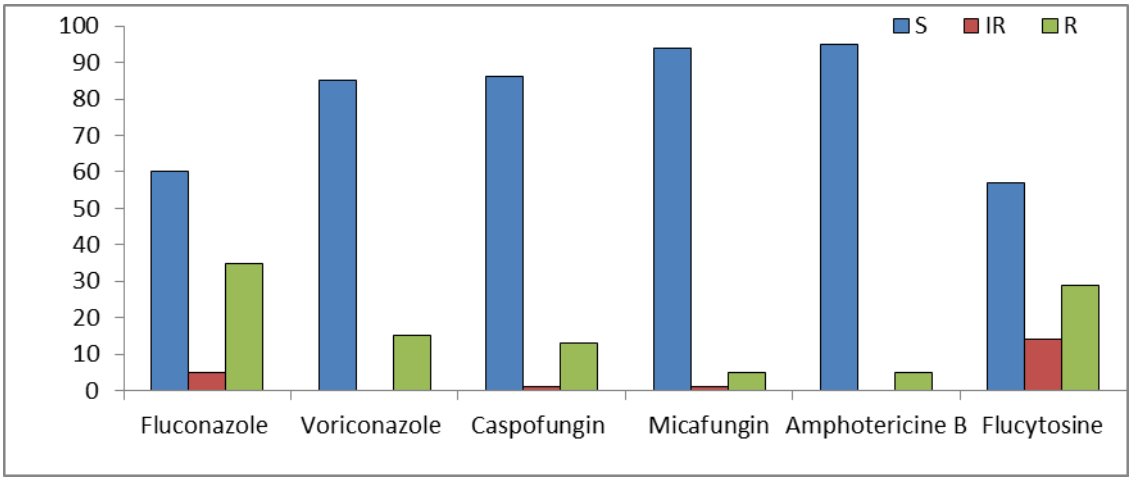


**Fig. 4:** Antifungal susceptibility pattern of isolated strains in the current study

*Antifungal susceptibility pattern of isolated yeasts:*

As shown in Table (4), the different yeast isolates (90 isolates) were tested for their sensitivity to six different antifungal therapeutic agents.

**Table 4: Antifungal susceptibility pattern of isolated yeasts:**

| | ***C.albicans*** **(n= 35)** | ***C.tropicals*** **(n= 34)** | ***C.glabrata*** **(n= 8)** | ***C.krusei*** **(n= 10)** | ***C.famata*** **(n= 2)** | ***C.parapsilosis*** **(n= 1)** |
|---|---|---|---|---|---|---|
| **Fluconazole** | | | | | | |
| S | 29 (82.9) | 26 (76.5%) | 2 (25%) | 3 (30%) | 0 | 0 |
| IM | 0 | 0 | 1(12.5%) | 4(40%) | 0 | 0 |
| R | 6 (17.1%) | 8(23.5%) | 5 (62.5%) | 3 (30%) | 2 (100%) | 1(100%) |
| **Voriconazole** | | | | | | |
| S | 30 (85.7%) | 34 (100%) | 7 (87.5%) | 9(90%) | 2(100%) | 1(100%) |
| IM | 0 | 0 | 0 | 0 | 0 | 0 |
| R | 5(14.3%) | 0 | 1 (12.5%) | 1(10%) | 0 | 0 |
| **Caspofungin** | | | | | | |
| S | 33 (94.3%) | 32 (94.1%) | 5 (62.5%) | 9(90%) | 1 (50%) | 1(100%) |
| IM | 0 | 0 | 1(12.5%) | 0 | 0 | 0 |
| R | 2(5.7%) | 2 (5.9%) | 2 (25%) | 1(10%) | 1 (50%) | 0 |
| **Micafungin** | | | | | | |
| S | 35(100%) | 34 (100%) | 5 (62.5%) | 9(90%) | 2 (100%) | 1(100%) |
| IM | 0 | 0 | 1(12.5%) | 0 | 0 | 0 |
| R | 0 | 0 | 2 (25%) | 1(10%) | 0 | 0 |
| **Amphotericin B** | | | | | | |
| S | 34 (97.1%) | 34 (100%) | 7(87.5%) | 9(90%) | 2 (100%) | 1(100%) |
| IM | 0 | 0 | 0 | 0 | 0 | 0 |
| R | 1(2.9%) | 0 | 1(12.5%) | 1(10%) | 0 | 0 |
| **Flucytosine** | | | | | | |
| S | 22 (62.9%) | 28 (82.4%) | 2(25%) | 1(10%) | 2 (100%) | 0 |
| IM | 2 (5.7%) | 4 (11.8%) | 5(62.5%) | 0 | 0 | 0 |
| R | 11 (31.4%) | 2 (5.9%) | 1(12.5%) | 9(90%) | 0 | 1(100%) |

Susceptibility of ten mould strains was evaluated as shown in Fig (5).

**Fig. 5:** Antifungal susceptibility testing of mould strain.

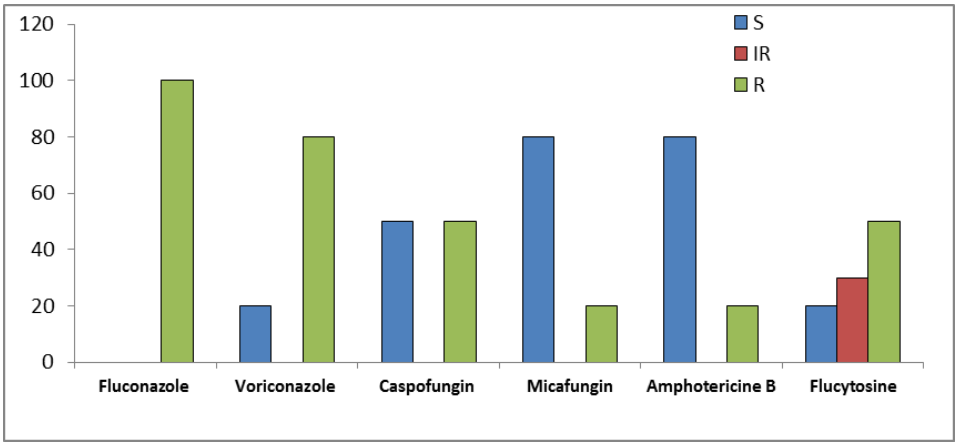


**Fig. 6:** Antifungal susceptibility pattern of identified Mould

As shown in Fig. (6), tested moulds were resistant to fluconazole (100%) and voriconazole (80%), while the highest sensitivity among mould strains was to Amphotericin B and micafungin (80%) and caspofungin (50%) respectively.

## DISCUSSION

The landscape of invasive mycoses is in a continuous evolution with significant consequences for their diagnosis and treatment. Invasive fungal infections (IFIs) are becoming a more important research topic as a result of their high contribution to morbidity and mortality, especially among immunocompromised patients.[12]

Antibiotic use (75%) and diabetes mellitus (25%) were the most important risk factors in our sample followed by steroid therapy (18%) and neutropenic fever (12%). similarly Ahmed et al.[11] found that antibiotic use (51.1%), neutropenia (28.9%), and diabetes mellitus (27.1 %) were the most popular risk factors .

It's a challenge to diagnose fungal infections. due to the lack of clear signs and symptoms , Colonization is difficult to discern from invasive disease, blood cultures are often negative, and patients are often unable to undergo invasive diagnostic procedures.[13] Microscopic analysis is a fast and relatively inexpensive method for diagnosing many fungal infections by detecting budding yeasts, pseudohyphae, and/or hyphae in samples.[14]

Just 21/120 (17.5%) of the samples in our study were found to be positive by direct microscopic examination.These findings were in line with those of Njunda et al.[18], who found that (11.5%) samples were positive for fungal infection by direct microscopic examination ,while Zarrinfar et al.[15] found that (4%) of 400 samples were positive by direct microscopic examination.

Microbiological culture is the cornerstone for diagnosing fungal infections among all diagnostic methods. Culture from a clinical sample has the benefit of revealing the real causative agent if positive. Furthermore, susceptibility testing is possible with culture.[16]

In our analysis, 100 (83.3%) of the 120 samples tested were positive for fungal growth when inoculated on SDA. These findings were close to those of Nasir et al.[17], who found that (68%) samples from HIV-positive patients tested positive for fungal infection. Gupta et al.[18] found that (54.5%) samples tested positive for fungi, which is lower than our findings. Also Taura et al.[19] recorded that (40%) of sputum samples tested positive for fungal infection.

It is noted that, *C. albicans* remains the predominantly isolated species. However, due to extreme immunosuppression, the use of broadspectrum antibiotics, and the empirical use of antifungal drugs, a move towards *non-albicans Candida* species has been noted.[20]

In present study, *Candida albicans* was the most frequently isolated species (38.9%), followed by *Candida tropicalis* (37.8%), *Candida krusei* (11.1%), and *Candida glabrata* (8.9 %).

Our findings are consistent with those reported by Khadka et al.[21] , who found that *Candida albicans* was the most common Candida species (56%) followed by *C. tropicalis* (20%), *C. glabrata* (14%) and *C. krusei* (10%) respectively. Also Talle et al.[22] , stated that *C. albicans* was the most widely described species. However, Ahn et al.[23], found that *C.parapsilosis* is the most common organism (53%) that causes fungal sepsis, followed by *C. albicans* (41%).

The identification of filamentous fungi is based on an accurate study of the macro- and microscopic characteristics of colonies grown on mycological media (SDA). The colony's size, colour, and form, microscopic visualisation of conidiophores and conidial heads, and the morphology, size, and colour of the conidia are all essential features for species identification.[24]

The macroscopic presence of the fungal colony on SDA and microscopic features in LCB stained wet mounts were used to identify filamentous fungi (10 isolates) in the current research. The most common isolated species were *A. flavus* (4%) and *A. niger* (4%), followed by *Penicillium spp* (2 %). These findings were consistent with those of Zarrinfar et al. [15] These findings were consistent with those of Zarrinfar et al.[15], who discovered that *A. flavus* was the most common causative agent among *Aspergillus* isolates, followed by *A. niger*, *A. fumigates*, *A.terreus*, and *Penicillium spp*. Also, according to Ahmed et al.[11], *A.flavus* was the most common isolated species (35.6%), followed by *A.niger* (32.1%).

The high incidence of *A. flavus* isolation in our patients may be attributed to the fungus's higher prevalence in the surrounding environment, such as air, water, or soil.

Antifungal susceptibility testing was used to diagnose antifungal resistance and to assess the best antifungal agent for a particular fungus. These approaches are used in clinical microbiology to determine the best treatment for a fungal infection and to determine the local and global epidemiology of antifungal resistance.[25]

In the current research, in-vitro susceptibility for yeast strains was determined using the Vitek 2 system, and disc diffusion method for mould strains. Amphotericin B (95% sensitivity) was found to be the most effective drug, followed by Micafungin (94% sensitivity), Caspofungin (86% sensitivity), Voriconazole (85% sensitivity), fluconazole (60), and flucytosine (57%). These findings are consistent with

those of Bustamante et al.[26],Who found that (98.0%) of the isolates were susceptible to amphotericin B. Also Khan et al.[27], found that nystatin and amphotericin B were the most important drugs for their isolates.

Antifungal resistance has recently been discovered in various Candida species.[28] Additionally, fungal strains isolated from immunocompromised patients have higher antifungal resistance due to the use of antifungals as prophylaxis.[29]

Fluconazole resistance was found in 35% of total isolates in our sample, and flucytosine resistance was found in 29%. This is consistent with the findings of Sheneef et al.[30] , who found that all isolates were Fluconazole resistant. Fluconazole resistance is a major concern since it is the most widely prescribed azole for suspected fungal infections, and drug resistance develops quickly during monotherapy.

## CONCLUSION

Invasive fungal infections are a significant cause of morbidity and mortality in immunocompromised patients, according to our report. Candida and Aspergillus species were the most common fungal pathogens found in the ICU of Assiut University Hospital.

In clinical microbiology laboratories, the chromogenic media and VITEK2 card system tend to be excellent alternative methods for yeast identification. Traditional recognition techniques, on the other hand, should be used in tandem. Brilliance Candida agar media had the best discriminating ability, being able to tell the difference between five of the seven Candida species studied. identification is mainly based on phenotypic features.

Antifungal susceptibility testing was extremely helpful in weeding out ineffective antifungals and allowing for better selection of the most effective drugs. For disseminated fungal infection, amphotericin B is the drug of choice.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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