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ORIGINAL ARTICLE

# Isolation of Cryptococcus Neoformans from Clinical Samples and Evaluation of Resistance to Antifungal Drugs

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***Key words:***
***Cryptococcus neoformans, antifungal resistance, disk diffusion, opportunistic fungi, Cryptococcus Differential Agar (CDA).***

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## ABSTRACT

***Background:*** *Cryptococcosis, a severe fungal infection primarily caused by Cryptococcus neoformans, presents substantial risks, particularly to immunocompromised individuals.* ***Objectives:*** *This study aimed to isolate Cryptococcus species from a range of clinical samples and assess their susceptibility to commonly used antifungal agents, containing nystatin, amphotericin B, itraconazole, voriconazole, and fluconazole.* ***Methodology:*** *A total of 240 clinical samples—including sputum, vaginal swabs, bronchoalveolar lavage, and cerebrospinal fluid (CSF)—were collected from patients in hospitals throughout Babylon Governorate, Iraq, during the period between November 2024 and March 2025. Cryptococcus isolates were cultured on Sabouraud dextrose agar (SDA) supplemented with chloramphenicol and were identified through India ink staining, urease testing, and Cryptococcus Differential Agar (CDA)media. Antifungal susceptibility was evaluated using the disk diffusion method, adhering to CLSI standards for accurate and standardized testing.* ***Results:*** *Out of 240 clinical samples, 28 (11.6%) were positive for cryptococcus species. The most common one among these isolates was C. neoformans that accounted for more than 75% of the samples, followed by Cryptococcus gattii (17.8%) and Cryptococcus laurentii (7.1%). Alarmingly, all isolates exhibited complete resistance to nystatin, amphotericin B, itraconazole, voriconazole, and fluconazole.* ***Conclusion:*** *These findings highlight a concerning prevalence of multidrug resistance, especially in Cryptococcus neoformans, underscoring an urgent need for enhanced antifungal stewardship. Furthermore, the results emphasize the importance of molecular surveillance to monitor resistance mechanisms and the necessity for novel antifungal therapies to address emerging challenges in treatment.*

## INTRODUCTION

Opportunistic fungi are those that are acquired through inhalation and can cause infection under certain conditions such as severe immunodeficiency, which includes people with AIDS, cancer, old age, immunosuppressive therapy, and those undergoing organ transplants (both blood and solid organs) [1].

*Cryptococcus*, an encapsulated basidiomycete yeast, most commonly affects the lungs and central nervous system and causes potentially life-threatening illness, Infection of the lungs occurs without any clinical symptoms in healthy individuals[2]. Cryptococcus's ability to grow at a human body temperature of 37°C and adapt to more extreme temperature conditions, such as those found in carrier animals as pigeons, whose body temperature is 42 ± 1.3°C lower than that of humans, is a major factor contributing to its success as a fungal pathogen [3].

*Cryptococcus* genus involves 70 species [4] but the species considered dangerous and causing diseases to humans and animals are C. *gattii* and *C. neoformans* [5], which are ubiquitous, saprophytic, round, basidiomycetous yeasts (5 to 10 µm) with a large heteropolysaccharide capsule (1 to 30 µm) [6]. *Cryptococcus neoformans* and *Cryptococcus gattii* can be distinguished from other pathogenic yeasts such as *Candida* by several characteristics including the presence of a polysaccharide capsule, melanin formation, and urease activity, all of which act as determinants of virulence [7]. *C. neoformans* infects the human central nervous system, it can cause pneumonia and meningitis and is responsible for substantial morbidity and mortality [8]. In addition to the central nervous system and lungs, muscles, skin, joints, bones, liver, kidneys, and other organs can also be affected [9] . *C. gattii* is closely associated with organic matter present in trees and decaying wood. It has a wide geographical distribution in many countries of the world still considered a primary pathogen, a life-threatening agent, and most infections occur in the northeastern region of Brazil [10].

Since no vaccine exists for cryptococcosis, pre-emptive therapy plays a crucial role in reducing the risk of cryptococcal meningitis among high-risk individuals. For localized cryptococcosis, treatment typically involves fluconazole, whereas severe and disseminated infections require amphotericin B in combination with flucytosine, followed by a step-down to fluconazole for continued management [11]. The efficacy of antifungal treatments varies significantly among Cryptococcus spp. strains, influenced by genetic and sample-specific factors and site of infection [12].

## METHODOLOGY

***Samples Collection:***

A total of 240 clinical samples were collected, including 110 sputum samples from patients with respiratory diseases, 80 vaginal swab samples, 36 bronchoalveolar lavage samples, and 14 cerebrospinal fluid samples (CSF), from patients attending Marjan Teaching Hospital, Dr. Saleh Al-Mukhtar Chest and Respiratory Center, Babylon Women's and Children's Hospital, and Imam Al-Sadiq Hospital in Babylon Governorate during the period from November 2024 to March 2025. The study included both sexes, and their ages ranged from 4 months to 85 years. Information of all patients was recorded using private data.

***Identification of Cryptococcus Spp isolates:***

To isolate *Cryptococcus spp*. from clinical samples, cultures were done on Sabouraud Dextrose Agar (SDA) supplemented with 0.05 mg/ml chloramphenicol. The plates were incubated at 37°C for 72 hours to allow fungal growth. Colonies suspected to belong to Cryptococcus species, were stained microscopically by India ink and examined a capsule. Positive colonies are transferred to Cryptococcus differential medium (CDA) to differentiate *cryptococcus species* based on color after incubation at 37°C for 36–48 hours. We also use chemical tests, including the urease assay, to confirm the purity of the isolate. The color change from yellow to pink indicates urease activity, which aids in species identification [13]

***Antifungal Susceptibility Test:***

Antifungal susceptibility testing was conducted using the agar diffusion (disk diffusion) method. *Cryptococcus species* were grown on Sabouraud dextrose agar for 48 hours. Colonies were suspended in 0.9% saline and adjusted to a turbidity equal to the 0.5 McFarland standard, achieving a concentration of $1\times10^6$ to $5\times10^6$ cells/ml. A sterile cotton swab was dipped into the suspension, excess fluid was removed, and the swab was streaked in three directions over Sabouraud dextrose agar plates, rotating the plate 60° between each streak to ensure even distribution[14].

After allowing the inoculated plates to dry for 5–15 minutes, antifungal disks (fluconazole 10 mcg, amphotericin B 100 units, itraconazole 50 mcg, voriconazole 1 µg, and nystatin 50 mcg) were placed on the agar surface. Plates were incubated under standardized conditions. Zones of inhibition were measured according to CLSI guidelines to interpret susceptibility. Zone diameter breakpoints were used to classify the isolates as susceptible (S), susceptible-dose dependent (S-DD), or resistant (R) for each antifungal tested [15]

**Ethical Considerations:**

Samples were collected after approval from the Ethics Committee, in accordance with Order No. 1888 issued by the Babylon Health Department on October 10, 2024.

## RESULTS

Out of 240 clinical samples were collected—comprising 110 sputum samples from patients with respiratory diseases, 80 vaginal swabs, 36 bronchoalveolar lavage, and 14 cerebrospinal fluid (CSF) samples— A total of 28 samples demonstrated positive growth of *Cryptococcus species*. This corresponds to an infection rate of 11.6% (Table 1).

**Table 1:** number and percentage of samples positive for *Cryptococcus* species

| Sample Type | Total Samples | Positive Samples | Percentage of Positive Samples |
|---|---|---|---|
| Sputum | 110 | 16 | 14.5% |
| Vaginal Swab | 80 | 7 | 8.75% |
| Bronchoalveolar Lavage | 36 | 2 | 5.5% |
| Cerebrospinal Fluid (CSF) | 14 | 3 | 21.4% |
| **Total** | **240** | **28** | **11.6%** |

***Identification of Cryptococcus species:***

On Sabouraud's Dextrose Agar (SDA), the *Cryptococcus* isolates displayed their characteristic creamy white colonies, indicative of their typical growth morphology. Microscopic examination after India ink staining revealed budding yeast cells. When India ink staining was performed, a bright halo surrounding the yeast cells became visible, indicating the presence of capsular material. Microscopic examination using India ink staining revealed budding yeast cells. Furthermore, The India ink staining revealed a bright halo around the yeast cells, signifying the presence of capsular material, a characteristic feature of *Cryptococcus* species (Fig. 1).

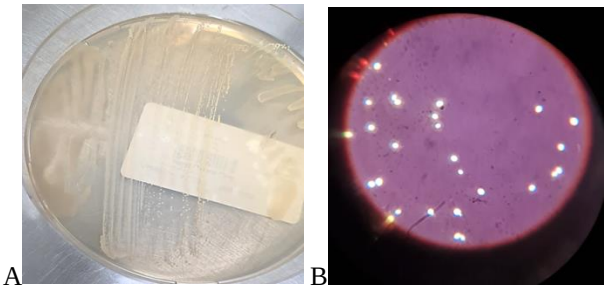

**Fig. 1**: [A] Macroscopic Visual of *Cryptococcus neoformans* colonies on Sabouraud Dextrose Agar, [B] Microscopic feature of *Cryptococcus neoformans* stained with India ink (40X), exhibiting thick polysaccharide capsule.

All isolates tested gave a positive result for urease activity, which is abundantly produced by *C. neoformans*. urease production in *C. neoformans* can be identified using urease test media, where a color alteration from yellow to pink or light purple (Fig. 2).

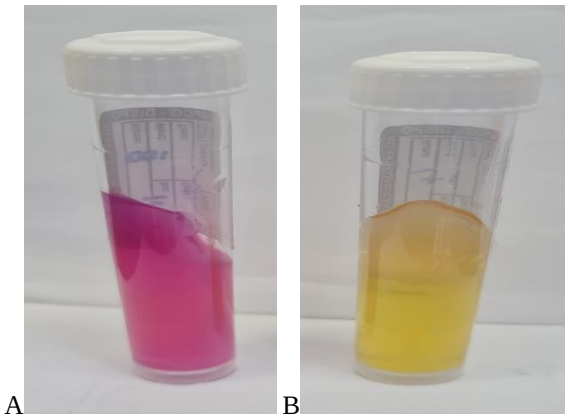

**Fig. 2**: [A] *C. neoformans* demonstrated urease activity, as evidenced by turning the Christensen's urea slant pink, [B] Urease negative.

Cryptococcus Differential Agar (CDA) was employed to distinguish between three *cryptococcus species*: *C. neoformans*, *C. gattii*, and *C. laurentii*. After incubation at 37°C for 7 days, *C. neoformans, C. gattii, and C. laurentii* displayed light blue, dry brown mucoid, and brown dry colonies, respectively Using this method, 21 isolates (75%) were identified as *C. neoformans*, 5 isolates (17.8%) as *C. gattii*, and 2 isolates (7.1%) as *C. laurentii*, confirming the distribution of species within the analyzed samples (Table 2 and Fig.3).

**Table 2: The number of *Cryptococcus spp*. isolates from different clinical sample.**

| ***Cryptococcus* species** | **Isolation No.** | **%** |
|---|---|---|
| *Cryptococcus neoformans* | 21 | 75% |
| *Cryptococcus gattii* | 5 | 17.8% |
| *Cryptococcus laurentii* | 2 | 7.1% |

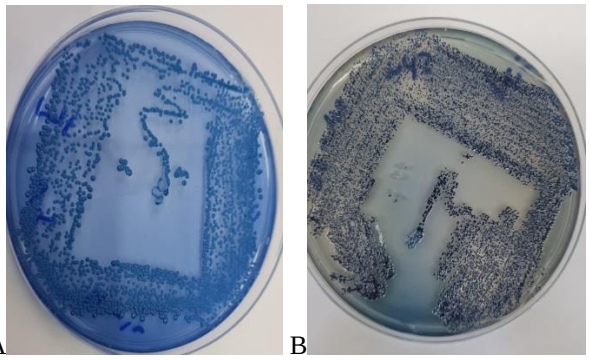

**Fig. 3**: illustrates Cryptococcus Differential Agar (CDA) after 7 days of incubation, showcasing: (A) The colonies of *C. neoformans* appeared as light blue and dry, (B) dry brown mucoid colonies of *C. gattii*.

***Antifungal susceptibility test of Cryptococcus species***

The antifungal susceptibility test for *Cryptococcus species* to fluconazole (FLC) 10 mcg, amphotericin B (AMB) 100 units, itraconazole (ITC) 50 mcg, voriconazole (VRC) 1 µg, and nystatin 50 mcg. The findings revealed that 100% of the Cryptococcus isolates tested displayed complete resistance to all five antifungal drugs: fluconazole (FLC), amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), and nystatin.

This highlights the alarming resistance exhibited by *Cryptococcus species* to these antifungal agents, as shown in (Fig. 4).

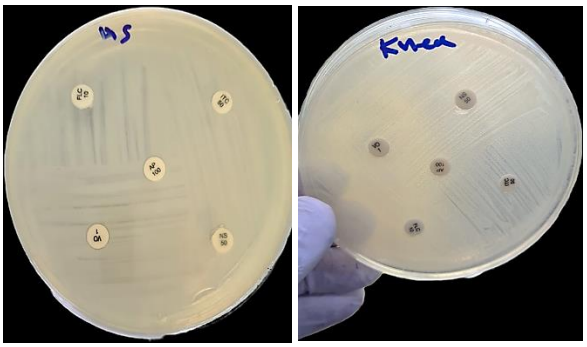

**Fig. 4:** *Cryptococcus species* demonstrated resistance, with no inhibitory zones observed, to all five antifungal agents tested: FLC, AMB, ITC, VRC, NS.

## DISCUSSION

The current study identified *Cryptococcus species* in 11.6% of 240 clinical samples collected from several hospitals in Babil Governorate, Iraq. The highest prevalence was observed in cerebrospinal fluid (CSF) samples (21.4%), followed by sputum samples (14.5%), vaginal swabs (8.75%), and bronchoalveolar lavage samples (5.5%). This distribution is consistent with available evidence that *Cryptococcus neoformans*

primarily infects immunocompromised individuals, particularly in cases of lung and central nervous system infections, such as cryptococcal meningitis. A study by Melhem et al. (2024) showed similar results, with C. neoformans being the dominant strain in infections in immunocompromised patients [16]. In China, a study by Wang et al. (2023) showed that C. gattii was prevalent among patients in tropical environments, highlighting geographic variation in the prevalence of the species[17].

Biochemical tests and microscopic analyses confirmed the capsular morphology and urease activity of the isolates, which are key virulence factors for *Cryptococcus species*. The application of Cryptococcus differential agar (CDA) and urease testing has been shown to be highly effective in distinguishing between species within the genus and verifying their identities, as also demonstrated in previous studies [1,18]. Baker & Casadevall (2023) showed that *C. neoformans* possesses several pathogenic factors, such as urease and melanin, that help the fungus evade the immune system, making the infection more difficult to treat [19]. This echoes the findings of studies such as Jones et al. (2017), which emphasized the importance of understanding the interaction of these factors with each other in order to improve treatment strategies and lead to the development of new drugs that directly target virulence mechanisms [20].

The alarming findings of widespread resistance demonstrated by all *Cryptococcus* isolates to the five tested antifungals: fluconazole, amphotericin B, itraconazole, voriconazole, and nystatin. This is consistent with the findings of numerous studies in this field. In a study conducted in Iran by Bandalizadeh et al. (2020), increasing resistance of *C. neoformans* to antifungal drugs such as fluconazole and azoles was observed [21]. This reflects the global trend of increasing drug resistance in cryptococcal fugi, especially in low-income countries. Moreover, some fungi have also shown resistance to some antifungals. In a study conducted by Jabber and Zghair (2024) analyzed antifungal resistance among *Candida* isolates from 125 cancer patients in Al-Najaf, Iraq. Among the 59 confirmed cases of *Candida* infection, 45 isolates were found to be sensory to both nystatin and fluconazole. In comparison, 14 isolates exhibited resistance to antifungal agents—two were resistant only to fluconazole, six exclusively to nystatin, and another six showed resistance to both drugs. This resistance was primarily linked to genetic factors, particularly the overexpression of resistance genes, such as fks, which significantly enhanced the fungi's ability to expel antifungal agents, reducing their effectiveness [22] .

Several factors may contribute to this pattern of resistance, including the overuse and misuse of antifungal drugs in both clinical and agricultural settings, genetic adaptation of fungal strains, and the emergence of novel resistance mechanisms. Research has shown that prolonged exposure to azoles, such as fluconazole, can induce upregulation of efflux pumps or lead to mutations in target enzymes [23]. Another study, highlighting the emergence of novel resistance mechanisms, suggests that *C. neoformans* may play a role in the development of novel resistance mechanisms, including cell wall changes or increased melanization, which helps the fungus evade the immune system and overcome the effects of antifungal drugs(19)

## CONCLUSION

The present study highlights the presence of *Cryptococcus neoformans* in Babylon province with an overall incidence rate of 11.6%. It is noted that *C. neoformans* is more common than other species, especially in immunocompromised patients. It is worth noting that all isolates of commonly used antifungals, such as fluconazole, amphotericin B, itraconazole, voriconazole, and nystatin, showed complete resistance, which is alarming. These results emphasize the urgent need for molecular monitoring of resistance mechanisms, continuous development of therapeutics, and improved management of cryptococcal infections in this region.

**Declarations:**

**Consent for publication:** Not applicable

**Availability of data and material:** Data are available upon request.

**Competing interests:** The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This manuscript has not been previously published and is not under consideration in another journal.

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