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# Molecular Characterisation of Virulence Factors in *Cryptococcus* spp. Isolated from Humans, Pigeons, and *Eucalyptus* Sources

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## Abstract

This study aimed to isolate and identify *Cryptococcus* species from three distinct sources: sputum samples of pigeon fanciers, dried pigeon droppings, and eucalyptus tree leaves. A total of 150 specimens were collected over a two-month period, comprising 50 samples each from human sputum, pigeon droppings collected across various areas of Baghdad, and eucalyptus leaves obtained from the Baghdad College of Veterinary Medicine. All samples were cultured on Sabouraud dextrose agar supplemented with chloramphenicol and incubated at 25°C for 2–3 days.

From the initial cultures, 20 isolates presumptively identified as *Cryptococcus* spp. were obtained: 6 isolates (12%) from human sputum, 9 isolates (18%) from pigeon droppings, and 5 isolates (10%) from eucalyptus leaves, giving an overall recovery rate of 13.3%. Molecular identification using PCR was employed to detect two key virulence genes: *CAP64* (associated with capsule formation) and *LAC1* (involved in melanin production). These genes were detected in 13 out of 14 confirmed *Cryptococcus* isolates (92.85%). Specifically, 75% of *C. neoformans* isolates from human samples carried these genes, while the *C. albidus* isolate lacked them. All *Cryptococcus* isolates from pigeon droppings and eucalyptus leaves tested positive for both virulence genes (100%).

**Keywords:** *Cryptococcus spp., virulence genes, CAP64, LAC1, pigeon droppings, eucalyptus leaves.*

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## Introduction

Opportunistic yeasts and filamentous fungi are responsible for three primary forms of mycotic infections: (1) superficial and cutaneous, (2) subcutaneous, and (3) systemic or deep mycoses (Rathore, et al., 2022; Zhao, et al., 2023; Kamal, & Al-Hadad, 2023; Mohammed, & Kamal, 2025). Numerous cases of cryptococcosis, candidiasis, and aspergillosis have been documented across the Americas, Europe, and Africa, confirming their global occurrence (De Hoog, et al., 2005; Jassem, et al., 2013; Mahmood, 2015). Since the 1980s, the increasing number of individuals with weakened immune systems has made opportunistic fungal infections a significant public health challenge (Francisco, de Jong, & Hagen, 2021;

Mukaremera, 2023; Mohammed, & Al-Gburi, 2023; Khalaf, & Rejah, 2024). The mortality rates associated with the three leading invasive fungal pathogens—*Aspergillus*, *Candida*, and *Cryptococcus*—range from 10% to as high as 90% (Steinbach, & Stevens, 2003; Filioti, Spiroglou, & Roilides, 2007; Longley, et al., 2008; Park, et al., 2009; Kadim, et al., 2019).

*Cryptococcus*, a fungal pathogen, causes cryptococcosis, particularly among immunocompromised individuals. The two main species implicated in human disease are *Cryptococcus neoformans* and *Cryptococcus gattii* (Morales-López, & Garcia-Effron, 2021; Black, et al., 2024). These organisms are found in various environmental reservoirs worldwide, with typical exposure occurring through contact with soil or avian droppings (Lin, Shiau, & Fang, 2015; Refai, El-Hariri, & Alarousy, 2017). Morphologically, these pathogens resemble encapsulated yeasts and are capable of causing serious infections (Diniz-Lima, et al., 2022; Ristow, et al., 2023; Khalaf, & Rejah, 2024). Individuals with underlying conditions such as AIDS, diabetes, chronic hepatic or renal illnesses, prolonged corticosteroid use, or those who have undergone organ transplantation are especially at risk (Diniz-Lima, et al., 2022; Kamal, & Al-haddad, 2022).

Virulence factors are broadly defined as components or characteristics of a pathogen that contribute to host damage (Chen, et al., 2022; Montoya, Magwene, & Perfect, 2021; Denham, et al., 2022). *C. neoformans* is known to produce a variety of virulence-associated enzymes, including proteases and lipases—similar to those produced by other microbial pathogens such as bacteria and fungi (Ghanem, & Sivasubramanian, 2021; Alegre-González, et al., 2021; Poplin, et al., 2024). Additionally, this species secretes urease, an enzyme involved in nitrogen metabolism by catalysing the breakdown of urea into $CO_2$ and ammonia. This enzyme plays a critical role in pathogenesis and is used as a diagnostic indicator for cryptococcosis (Rizzo, et al., 2021; Yu, et al., 2021; Yang, et al., 2022; Mohammed, & Kamal, 2025).

Among the most studied virulence factors in *C. neoformans* are its polysaccharide capsule and melanin pigment. These two components are not only vital for the pathogen's virulence but also provide protection against host immune responses (Baddley, et al., 2021; Wang, et al., 2022; Huang, et al., 2024).

## Methodology

### *LAC1* Gene Primers

**Table 1. *LAC1* Gene Primers Used in the Study of *Cryptococcus* spp.**

| Primer | Sequence | Primer sequence | Tm (°C) | GC% | Size of Product (bp) | |
|---|---|---|---|---|---|---|
| *LAC1* *C. neoformans* | F | 5'- AGAAGGGAAGGAAGGTGATG -3' | 60.3 | 50 % | 480bp | Musavinasab-Mobarakeh, Shams-Ghahfarokhi, & Razzaghi-Abyaneh, (2021) |
| | R | 5' TATACCTCACAACCGCCAAT -3' | 57.8 | 41 % | | |
| *LAC1* *C. gattii* | F | 5'- AACATGTTCCCTGGGCCTGTG -3' | 60.3 | 50 % | 469bp | |
| | R | 5' ATGAGAATTGAATCGCCTTGT -3' | 57.8 | 41 % | | |
| *LAC1* *C. albidus* | F | 5'- AGAAGGGAAGGAAGGTGATG -3' | 60.3 | 50 % | 480bp | |
| | R | 5' TATACCTCACAACCGCCAAT -3' | 57.8 | 41 % | | |

### *CAP64* Gene Primers

**Table 2. *CAP64* Gene Primers Used in the Study of *Cryptococcus* spp.**

| Primer | Sequence | Primer sequence | Tm (°C) | GC% | Size of Product (bp) | |
|---|---|---|---|---|---|---|
| *CAP64* *C. neoformans* | F | 5'- CTCTACGTCGAGCAAGTCAAG -3' | 60.3 | 50 % | 559bp | Imanishi-Shimizu, |
| | R | 5' TCCGCTGCACAAGTGATACCC -3' | 57.8 | 41 % | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *CAP64* *C. gattii* | F | 5'-CTGATCACACCGATCTCGTCATTCT -3' | 60.3 | 50 % | 833bp | et al. (2021) |
| | R | 5' GATCAGGCCTCACAAGGAT -3' | 57.8 | 41 % | | |
| *CAP64* *C. albidus* | F | 5'- CTCTACGTCGAGCAAGTCAAG -3' | 60.3 | 50 % | 559bp | |
| | R | 5' TCCGCTGCACAAGTGATACCC -3' | 57.8 | 41 % | | |

### Preparation of Culture Media

#### Sabouraud Dextrose Agar (SDA):

To prepare the medium, 65 grams of SDA powder were dissolved in one litre of distilled water. Chloramphenicol was added at a concentration of 0.05 g/mL to inhibit bacterial contamination. The mixture was stirred continuously while heating and sterilised in an autoclave at 121°C and 15 psi for 15 minutes. After cooling to approximately 50°C, the sterile medium was poured into petri dishes and incubated at 37°C for 24 hours before storage at 4°C.

### Samples Collection

#### Human Sputum Samples:

Sputum samples were obtained from pigeon breeders of different age groups. The samples were transported aseptically to the Mycology Laboratory of the Zoonotic Disease Research Unit. Each sample was streaked onto SDA supplemented with chloramphenicol (Staib, et al., 1987). Suspected yeast colonies were stained with India ink and lactophenol cotton blue, followed by a urease test. Further identification was confirmed using specific media.

#### Pigeon Droppings:

Fifty samples of pigeon droppings were collected from various pigeon shelters and placed in sterile plastic bags. Approximately 20–30 grams of each sample were weighed and diluted in saline (0.9%) containing 200 mg/L chloramphenicol to achieve a 1:10 dilution. The mixture was shaken for 30 minutes and left to settle. From the supernatant, 0.5 mL aliquots were streaked onto SDA. Confirmation involved staining (India ink, lactophenol cotton blue), urease testing, and growth on BSA and CDA media (Zarrin, Met al., 2010).

#### Eucalyptus Leaves:

Fifty eucalyptus leaf samples were collected from locations in Baghdad and placed in sterile plastic bags. Samples were transported on ice to the Zoonotic Diseases Unit, College of Veterinary Medicine. Two grams of leaves were rinsed in sterile water and then immersed in 20 mL of sterile saline with 10 mg/mL chloramphenicol. The mixture was homogenised for four minutes, then left at room temperature for 30 minutes. A loopful of the supernatant was streaked onto SDA plates, which were incubated at 30°C for 48 hours (Elhariri, et al., 2016).

## Results and Discussion

Colonies suspected to be *Cryptococcus* species were observed on Sabouraud Dextrose Agar (SDA). These isolates displayed a creamy texture, convex elevation, and a glistening appearance, often exhibiting a pale off-white to beige hue. After incubation at 25°C and 30°C for 2–3 days, the mucoid appearance of the colonies became evident, which is attributed to the production of a polysaccharide capsule, as illustrated in Figure 1.

These observations are consistent with earlier reports by Washington, et al. (2024) and Khanal, Sharma, & Deb (2002), who noted that mucoid colonies are often the first indicator of *Cryptococcus* presence. The

colonies are typically fast-growing, soft, glistening, mucoid or creamy in appearance, smooth, convex, and slightly pink, tan, or yellowish-brown on solid media—a finding further supported by Washington, et al. (2024); Okagaki, et al. (2010) and Firacative, Trilles, & Meyer (2021). Isolates from various sources (human, pigeon droppings, and eucalyptus leaves) shared morphological traits: spherical, encapsulated, and non-myceliated cells. Notably, *C. neoformans* is characterised by a distinct mucinous capsule, especially when incubated at 30°C, which also supports compact basidiospore formation as noted by Karkowska-Kuleta, Rapala-Kozik, & Kozik (2009) and Wang, et al. (2024).

**Figure 1. Suspected *Cryptococcus* spp. Appeared within 2-3 Days of Incubation in 25°C on SDA**

**Table 3. Primary Isolation of *Cryptococcus* sp. from Human and Natural Habitat**

| Sources of sample | No. of samples | Primary isolation in lab | |
|---|---|---|---|
| | | No. of +ve | % |
| **Human** | 50 | 6 | 12 |
| **Droppings** | 50 | 9 | 18 |
| **Eucalyptus leaves** | 50 | 5 | 10 |
| **Total** | 150 | 20 | 13.3 |

**Table 4. Positive Results of Suspected *Cryptococcus* spp. Isolates in Stains, Selective Differential Media and Biochemical Test**

| Sources of sample | Stains | | Selective differential media | | | | | | Biochemical test |
|---|---|---|---|---|---|---|---|---|---|
| | LPCB | INDIA INK | BSA | CDA | BHIA with cycloheximide | BHIA with methyldopa | EA | ELA | Urease test |
| **Human (6)** | 6 +ve | 3 +ve | 3 +ve | 4 +ve | _ | 6 +ve | 5 +ve | 6 +ve | 6 +ve |
| **Droppings (9)** | 9 +ve | 6 +ve | 6 +ve | 6 +ve | _ | 9 +ve | 9 +ve | 9 +ve | 4 +ve |
| **Eucalyptus leaves (5)** | 5 +ve | _ | _ | 5 +ve | _ | 5 +ve | 5 +ve | 5 +ve | 4 +ve |

## Molecular Detection of Virulence Factors in *Cryptococcus* spp.

Out of 14 suspected *Cryptococcus* spp. isolates—four from humans, six from pigeon droppings, and four from eucalyptus leaves—a conventional PCR assay was conducted to confirm molecular identity and detect two key virulence genes: **CAP64** (responsible for capsule formation) and **LAC1** (involved in melanin production).

**Table 5. Virulence gene in *Cryptococcus* spp.**

| Sources of sample | Spp. and NO. of positive isolates | Gene | | | |
|---|---|---|---|---|---|
| | | *CAP64* | | *LAC1* | |
| | | NO. | % | NO. | % |
| Human sputum | *C. neoformans* (3) | 3 | 100 | 3 | 100 |
| | *C. albidus* (1) | 0 | 0 | 0 | 0 |
| Pigeon dropping | *C. neoformans* (6) | 6 | 100 | 6 | 100 |
| Eucalyptus leaves | *C. gattii* (4) | 4 | 100 | 4 | 100 |
| Total | 14 | 13 | 92.85 | 13 | 92.85 |

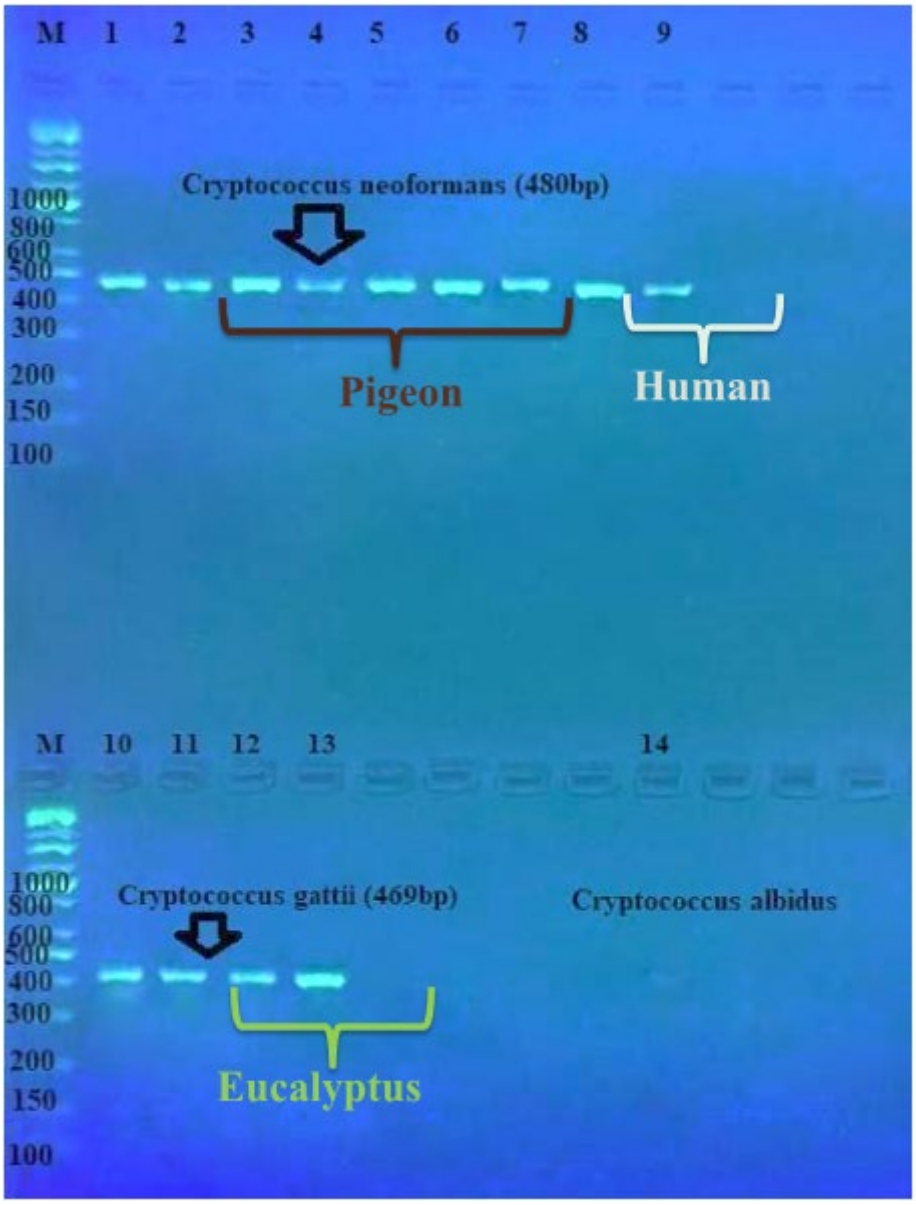


**Figure 2. Gel Electrophoresis for PCR Product of *LAC1* Gene, (Agarose 2%, at 70 volts, 60min.), M: DNA ladder (10000-100bp).Visualized under U.V light after Staining with Red Safe, (1-6) Represent *C. neoformans* from Pigeon Dropping, (7-9) *C. neoformans* from Human Sputum, *C. albidus* (14) from Human Sputum was Lack this Gene have Product size 469bp, (10-13) *C. gattii* from Eucalyptus Leaves with Size of Product 480bp**

PCR results revealed the presence of both **CAP64** and **LAC1** genes in 13 out of the 14 isolates, with *C. albidus* being the only isolate lacking both genes (Table 5). These findings corroborate previous studies (Ito-Kuwa, et al., 2007; Rodrigues, et al., 2015), which documented the detection of the capsule-encoding CAP64 gene and the LAC1 gene responsible for melanin synthesis in *C. neoformans* and *C. gattii* isolates from similar environmental and clinical sources (Meyer, et al., 2009; Specht, et al., 2024; Khalaf, & Rejah, 2024).

The **LAC1** gene, which governs melanin pigment production—a known virulence factor—was detected in all *C. neoformans* and *C. gattii* isolates but absent in *C. albidus*, as also reported by Kassi, et al. (2016); Samarasinghe, et al. (2018) and Wang, et al. (2022). PCR assays identified LAC1 at 469 bp in *C. neoformans* and 480 bp in *C. gattii*, which aligns with the findings of Ito-Kuwa, et al. (2007) and Kadim, et al. (2019).

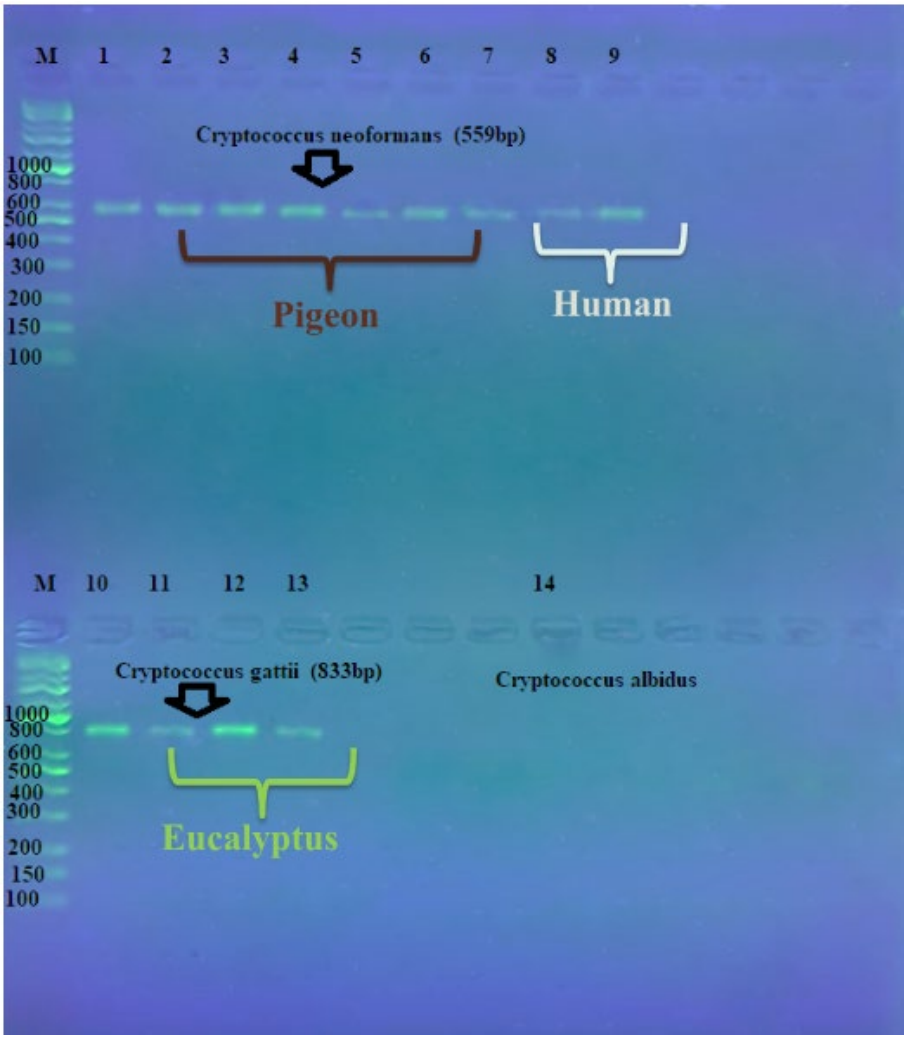


**Figure 3. Gel Electrophoresis for PCR Product of (CAP64), (Agarose 2%, at 70 volts, 60min.), M: DNA Ladder (10000-100bp).Visualized under U.V Light after Staining with Red Safe, (1-6) Represent *C. neoformans* from Pigeon Dropping, (7-9) *C. neoformans* from Human Sputum, *C. albidus* (14) from Human Sputum was Lack this Gene have Product Size 833bp, (10-13) *C. gattii* from Eucalyptus Leaves Size of Product 559bp.**

Similarly, the CAP64 gene, integral to the formation of the polysaccharide capsule and directly associated with the pathogenicity of *Cryptococcus* spp., was present in all *C. neoformans* and *C. gattii* isolates but absent in *C. albidus* (Figure 3). These findings are supported by Wilder, et al. (2002); Okabayashi, Hasegawa, & Watanabe (2007) and Imanishi-Shimizu, et al. (2021), who highlighted the role of CAP64 in virulence. However, these results contrast with those of Okabayashi, et al. (2005), who reported the lack of CAP64 gene expression in some *C. neoformans* isolates. PCR amplification of CAP64 showed band sizes of 833 bp in *C. neoformans* and 559 bp in *C. gattii*, while *C. albidus* exhibited no amplification.

## Conclusions

CAP64 and LAC1 virulence genes were detected in all clinical and environmental isolates of *C. neoformans* and *C. gattii* using conventional PCR, confirming their pathogenic potential. In contrast, *C. albidus* lacked both genes, suggesting a lower virulence profile.

Phylogenetic analysis demonstrated a 99% genetic similarity between the studied isolates of *C. neoformans* and *C. gattii* and their respective reference strains. *C. albidus* also showed a perfect match with known reference sequences, indicating accurate species identification.

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