Journal of Pioneering Medical Sciences
Received: November 15, 2025 | Accepted: January 02, 2026 | Published: February 05, 2026
Volume 15, Issue 01, Pages 165-169

DOI https://doi.org/10.47310/jpms2026150123

Molecular Study of Lycopene Effect on Clinical Isolates of *Cryptococcus Neoformans*

Abbas M. Ammari[1*]
[1]Department of Forensic Sciences, College of Science, University of Diyala, Iraq

Author Designation: [1]Assistance Professor

*Corresponding author: Abbas M. Ammari (e-mail: abbas@uodiyala.edu.iq).

Abstract **Background:** In Iraq, Cryptococcosis is the most common causes of invasive fungal infections. *Cryptococcus neoformans* appears a highly resistance toward antifungal treatments. Thus, this study revealed the correlation between this yeast and different infections as well as detect the Lycopene effect as an antifungal. **Objectives**: To detect the effect of Lycopene on resistant *C. neoformans* isolates that were isolated from different infections. **Materials and Methods:** One hundred swabs were collected from patients with respiratory infections, burns and meningitis which distributed as (40, 40 and 20) samples, respectively. The fruits of Tomatoes (*Lycopersicon esculentum* L.) were collected during the summer from local markets in Baghdad. All the clinical samples were cultured directly onto Sabouraud′s Dextrose agar (SDA) medium and incubate of 37°C for 7 days with daily examination. Lycopene was alcoholic extracted and purified using HPLC method. DNA of six *C. neoformans* isolates were taken away by Geneaid DNA extraction kit, depended on manufacturer′s instructions. Gene sequencing of the CAP60 gene was done to detect substitution mutations for *C. neoformans* that were treated and untreated with lycopene. **Results:** The positive percentages of *C. neoformans* among patients were (10.0%, 7.5% and 10.0%) for patients with respiratory infections, burns and meningitis, respectively. The macroscopic appearance of *C. neoformans* was ranged from whitish to creamy colored colonies with spherical, mucoid and smooth yeast cells. Microscopic examination of the yeast cell was characterized with gelatinous capsule surrounding. Molecular results revealed that the molecular weight of the CAP60 gene was 600 bp., The results of CAP60 gene sequencing indicate that two isolates treated with lycopene have more than one substitution mutation. **Conclusion:** the gene sequencing of the CAP60 gene revealed that the treated isolates with lycopene had more substitution mutations than the untreated isolates.

Key Words *Cryptococcus Neoformans*, Cryptococcosis, Lycopene, Antifungal Activity, CAP60 Gene, Molecular Characterization

INTRODUCTION

Cryptococcosis is the most common cause of invasive fungal infections, especially cryptococcal meningitis [1]. Since the 1970s, Cryptococci species of Gatti and neoformans are the commonest problematic Cryptococcosis in humans, especially [2]. Meningitis caused by Cryptococcus is reported to have a significant attributable mortality and morbidity among immunocompromised patients, especially in association with AIDS [3]. Cryptococcus is a yeast belonging to basidiomycetes. This genus has more than 30 species are sharing in many environmental features; they differ in characteristics due to the geographic distributions, hosts and their immune state and clinical features [4]. The patients with cryptococcal meningitis experience headache, fever, cranial problems, memory loss and irritation. These symptoms may be more acutely or less as absence of headache [5,6]. Cutaneous cryptococcosis is rare infection and classified as third commonest expressing of cryptococcus infections, it occurs either associated with another skin disease or by direct inoculation of this yeast. The symptoms of cutaneous cryptococcosis interacts with many skin lesions. Therefore, it needed to culture of skin biopsy for adequate diagnosis [7]. The treatment of cryptococcosis based on deficiency of virulence factors, as well as using of anti-cryptococcal drugs and assessment their activity by molecular study of virulence genes of causative agents of cryptococcosis and find alternative treatments for resistance isolates [8].

Lycopene is a carotenoid compound, it occurring in nature in some red colored plants like tomatoes, pink grapefruit,

watermelon, papaya and guava [9]. The human cell unable to synthesize lycopene. Thus, it supplemented through the daily diet and the age of humans is directly proportion with their needed for lycopene and the deficiency of this carotenoid association with some pathological problems such as cutaneous, cardiovascular and neurological diseases [10,11].

METHODS

Samples Collections

One hundred samples as total were collected from patient (males and females) whom admitted different teaching hospitals in Baghdad province for a year period (2023). The distribution specimens were possessed from patient respiratory infections and burn (40 each) and 20 from patients with meningitis.

The fruits of Tomatoes (*Lycopersicum esculentum*) were possessed during summer of locally market at Baghdad, Iraq. Firstly, the fruits lotion using distill water and sterilized using ethanol (70%). Next, they dried using oven with two periods (at 90° C for 90 minutes and at 60° C for 240 minutes). Then, the fruits were cut to cubic pieces with dimensioned (10×10×10) mm^3 and grinded in according to [12,13]. After that, the grinded fruits were packed in darkled test tubes and kept at room temperature until chemical extraction of lycopene.

Identification of *Cryptococcus Neoformans*

Cultural Identification: All the collecting specimens were streaked directly onto SDA medium with incubation at 37°C for seven days with daily examination. For microscopic examination, a loopful of *C. neoformans* colonies were placed onto sterilized slide and added a drop of Indian ink on the yeast with microscopic examination under (40x) to reveal capsules presence [6,14]. The isolated yeasts were macroscopic and microscopic identified based on their morphological characteristics [15].

Chemical extraction of Lycopene

The extraction of lycopene was performed according to [16]. Ten grams of dried tomatoes and put in darkled tubes to prevent light oxidation. Next, 100 ml from ethanol were added with shaking with using vortex for 30 minutes. Then, the tubes were left for 2 minutes and separate lycopene with filter solution under vacuum. After that, the extracted lycopene was place in a sterilize Petri dish and dried using anhydrous calcium chloride by vacuum desiccators.

Lycopene Purification

High performed liquid chromatography (HPLC) was used for Lycopene purification and as mentioned in [17]. The purification circumstances of HPLC were: columns were supplied they Chromoleth RP–C18A, 4.0×10 mm, injection volumes (40 µls) at 25°C, flows range (1cm^3/min), waves length (470 nm), solvents which used dimethylforomide (50%) and acetonitril (30%).

Detection of Lycopene Effect as Antifungal

The antifungal effect of purified lycopene was done using well diffusion technique [18,19]. Firstly, the isolates of *C. neoformans* were streaked onto Muller Hinton Agar medium. Next, the wells (5 mm in diameter) were done onto using a sterilized cork borer. After that, one hundred microliters of purified lycopene with concentrations (10,20,40 and 80 mg/ml) were spread into the wells. Finally, the cultured Petri dishes were incubated at 37°C for 24-48 hours.

Molecular Identification

DNA of six isolates of *C. neoformans* were possessed by Geneaid DNA extraction kit, Thailand, based on instructions of manufacture. CAP60 gene detected by forward primers CAP60F: 5`-GCAGCGGCTTGCCATTCGTG-3` and reversed primers (CAP60R: 5`-AGTCCGTGGAGGCGTGGTCA-3`) [15]. PCR amplifications of CAP60 gene were done in 25µl containing 3 µL of *C. neoformans* DNA, 10µl Taq Pol PCR Pre master Mix ,2µls of each primer (20pmol) and 8 µls of nucleases free water were add onto tubes till achieve 25 µL. PCR was done by thermos-cycler (Amersha, Sweden) depending on PCR program revealed by Alarousy *et al.* [16]. In a brief, the amplification of CAP60 gene of *C. neoformans* was done with initial denaturations at 94ºC for 4 mins follow 30 cycles PCR. Denaturation at 94ºC for 60 secs, annealings at 50ºC for 45 secs, extensions at 72ºC for 2 minutes and thermal cycles were terminated by final extensions at 72 °C for 5 mins. Nucleases free water use as a control; a 1.5 % agarose gel was used to analyze PCR product of CAP60 gene after it stained with ethidium bromide and detected using UV transilluminator. To reveal the genetic alterations of single nucleotide base that replaced by another, gene sequencing method was done for detection of substitution mutations of CAP60 gene for treated and untreated *C. neoformans* with lycopene.

The isolates were sent to Microgen company (South Korea) for CAP60 gene sequencing in both direction and all the reference nucleotide sequences of this gene from (www.ncbi) and aligned using MEGA4 program.

RESULTS AND DISCUSSION

Percentage of Morphologically Identified *Cryptococcus Neoformans*

The identification of *C. neoformans* isolates was based on the macroscope and microscopic examinations. Macroscopic examination of *C. neoformans* isolates that were cultured on SDA media incubated at 37°C for 48 hours appears as whitish to creamy in colour with spherical, mucoid and smooth yeast cells. Whereas, Microscopic examination appeared Globule to ovoid budded yeast cells with surrounding by a gelatinous capsule, as in Figure 1(a,b). The positive percentages of *C. neoformans* among patients was 9% (9 out of 100) as shown in Table 1. This result agreed with Inaam *et al.* [20] and Al-Temimay and Esraa [21]. *Cryptococcus neoformans* is an opportunistic yeast with

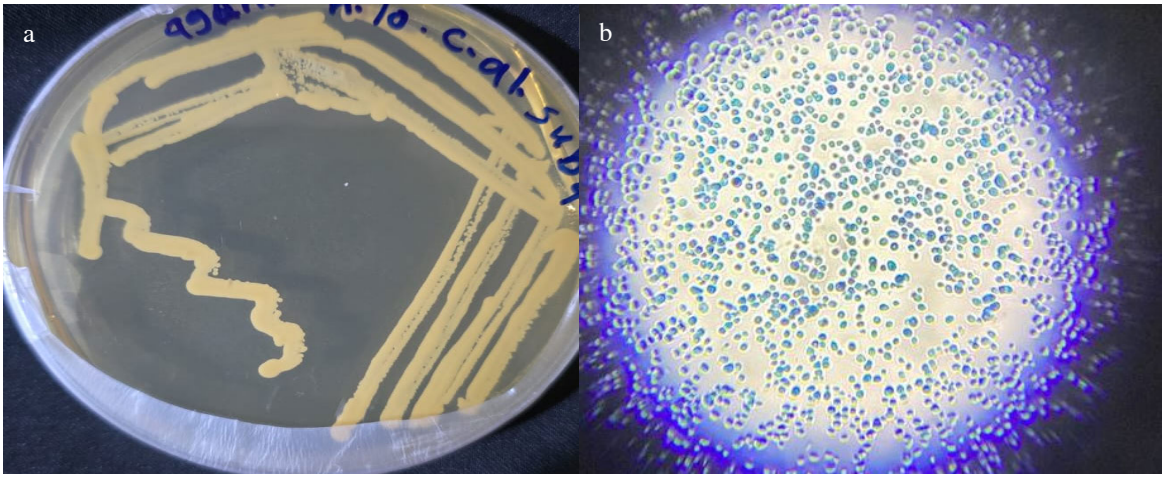

Figure 1(a,b): (a) Macroscopic features of *C. neoformans* colonies cultured on SDA medium and (b) Microscopic features of *C. neoformans* yeast stained with LPCB (40X)

Figure 2: Effect of purified lycopene on *C. neoformans* isolates

Table 1: Percentage of *C. neoformans* isolates concerning types of infection

Type of infections	Positive (%)	Negative (%)	Total (%)
Burns	3 (7.5%)	37 (92.5%)	40 (100.0%)
Respiratory infections	4 (10.0%)	36 (90.0%)	40 (100.0%)
Meningitis	2 (10.0%)	18 (90.0%)	20 (100.0%)
Total	9 (9.0%)	91 (91.0%)	100 (100.0%)

higher causative agent of morbidity and mortalities in immunocompromised than immunocompetent patients [22].

Effect of Purified Lycopene on *Cryptococcus Neoformans* Isolates

Three out of nine isolates of *C. neoformans* (33.3%) were sensitive to purified lycopene (40 mg\ ml) with ascending inhibition zones (4,9 and 13) mm. Harishawi *et al.* [18] found that the inhibition zones (6,7,8 and 9) mm, respectively, for

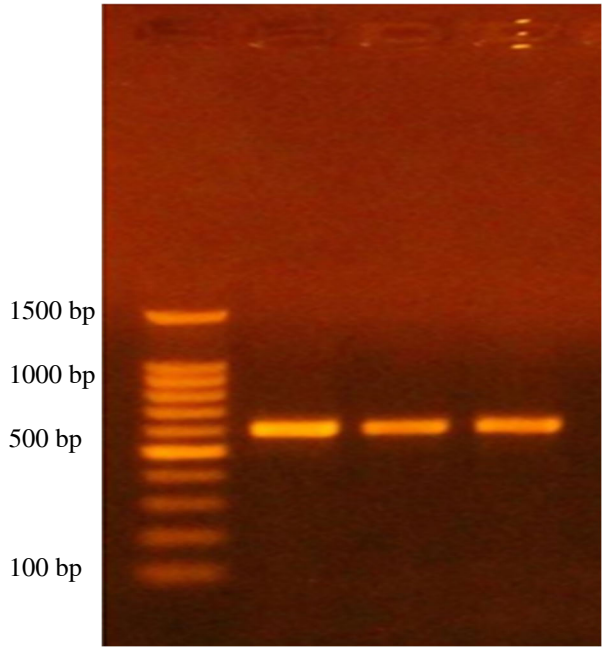

Figure 3: Gel electrophoresis of CAP60 gene product for *C. neoformans* using 2% agaroses gel at 6V/cm for 60 minutes. M: 100 DNA ladder, lanes 1-3: uniplex PCR products

C. neoformans as shown in Figure 2. The active compounds of lycopene with structural and chemical structures had excessive antimicrobial and biological activities [20].

Molecular Detection of the CAP60 Gene of *Cryptococcus Neoformans* Isolates

The molecular weight of CAP60 gene was 600 bp., this was indicated sign for successes reaction, as in Figure 3. After performing of the alignment between the amino acid reference sequences of CAP60 gene of *C. neoformans* and amino acids sequences of three isolates treated with lycopene and three isolates untreated with lycopene, two isolates of that treated with lycopene were mutant which

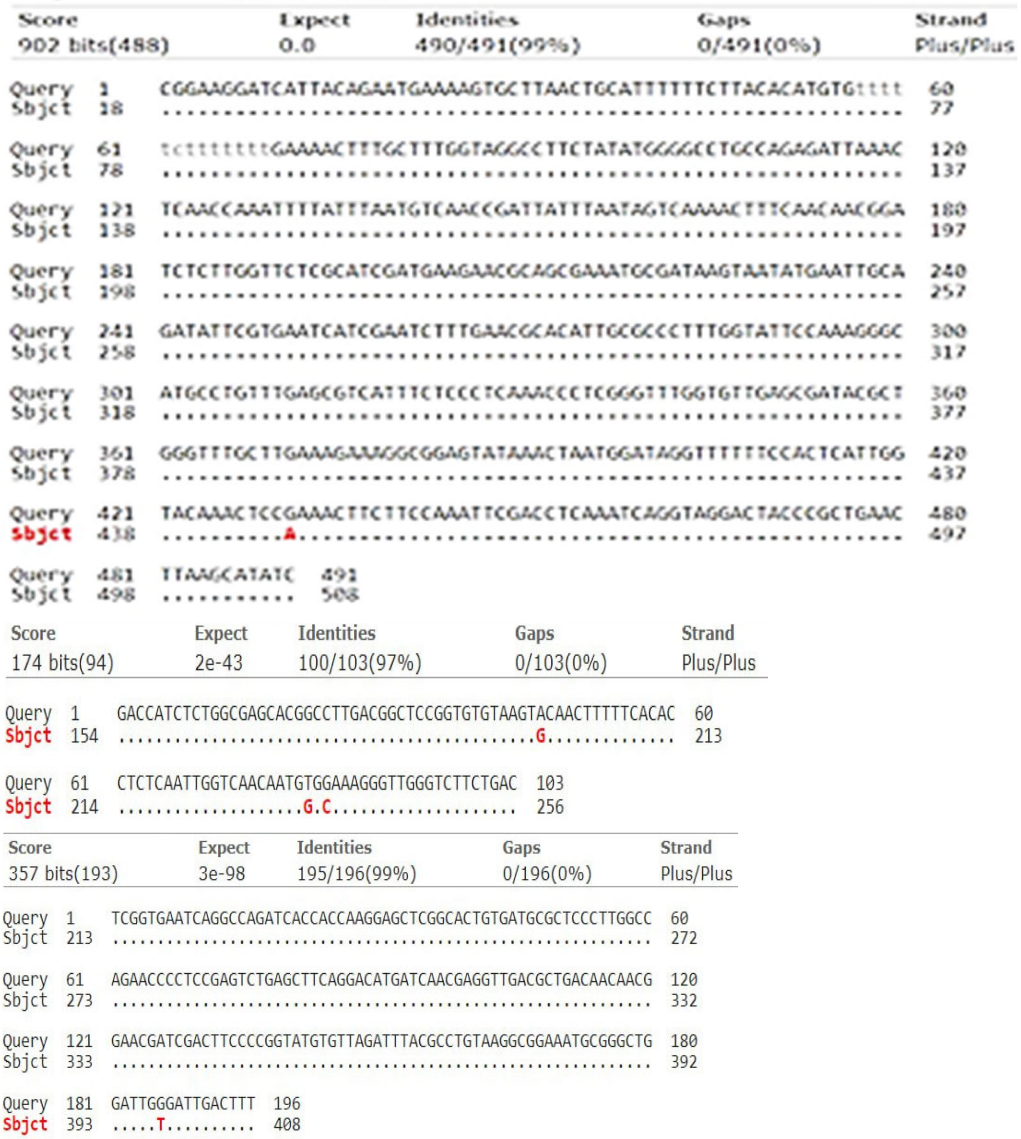

Figure 4: The substitution mutations of *C. neoformans* isolates that were treated with purified lycopene extract.

have more than one substitution mutations, like in Figure 4. There are no published results to compare with it. *C. neoformans* isolates require the CAP60 gene for capsule formation, which plays vital roles in isolates' virulence [8].

CONCLUSIONS

This study concluded that the gene sequencing of the CAP60 gene revealed that the treated isolates with lycopene had more than substitution mutations.

Ethical Statement

The information form of this study was confirmed by local ethic committee based on the decision 1828 in 12\1\2024.

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