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Isolation and Molecular Analysis of Cryptococcus laurentii from Pigeon Feces in Urban and Suburban Regions

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KEYWORDS
Biofilm, Cryptococcus laurentii, Multiplex PCR, Pigeon droppings

ABSTRACT
It was reported that invasive fungal infections result in significant morbidity and mortality rates, particularly in immunocompromised hosts. Although pigeon droppings might be a possible vector for the transmission of pathogenic yeasts, The goal of this study was to isolate and identify Cryptococcus species using genetic and phenotypic methods. The results were then confirmed by doing several tests on pigeon droppings. In this research paper, two hundred samples of pigeon droppings were collected from fifty-three diverse places in Erbil city and its suburbs between November 2021 and October 2022. It has been experimentally found that among these two hundred samples, a total of fifteen isolates of Cryptococcus were obtained from pigeon droppings in the aforementioned places. In addition, Cryptococcus laurentii was identified by culturing on Sabouraud Dextrose Agar (SDA) and Niger Bird Seed Agar (NBSA) based on capsule formation, biochemical methods using VITEK, Urease test, and Phenol oxidase test, and then confirmed by a molecular technique such as DNA extraction and PCR amplification of ITS region sequence by using universal primers. The sequence data of C. laurentii accession number of nucleotide OR509919 have been provided by GenBank available on the NCBI website. In the biofilm formation method, C. laurentii isolates produced biofilms according to both phenotypic and molecular methods in the biofilm formation method, C. laurentii isolates produced biofilms, according to both phenotypic using Congo Red Agar media (CRA), Microtiter Plate Approach (MTP) and molecular methods for amplification of the ALS1 and HWP1 genes. It has been concluded that the utilization of the multiplex-PCR technique is highly suggested as a technology that offers enhanced detection of Cryptococcus spp. with high sensitivity and specificity.

I. INTRODUCTION

Avian feces are recognized as a repository for several pathogenic and opportunistic organisms (Chee and Lee, 2005). The deadliest opportunist yeast, Cryptococcus neoformans, is thought to be carried widely by pigeons (Soltani, et al., 2013; Costa, et al., 2010). Furthermore, it is widely acknowledged that this particular entity serves as a reservoir for zoonotic yeasts and various other dangerous fungi (Medina, et al., 2017). In immunocompromised people, especially those with AIDS, Cryptococcus yeast is a major infectious agent. The two most common species of Cryptococcus, C. neoformans, and C. gattii, cause the disease (Casadevall, et al., 1998), in recent decades other species of Cryptococcus including C. laurentii, C. albidus, C. uniguttulatus, C. luteolus, C.adeliensis yeast have been known to cause infections in humans and animals (Khawcharoenporn, et al., 2007).

Encapsulated yeasts of the genus Cryptococcus are commonly found in nature (Perfect and Casadevall, 2002) and are often linked to birds and their droppings (Byrnes, et al., 2011). The sources of uncommon Cryptococcus spp. in the environment are exceptionally diverse, encompassing air, water, vegetation, food, and soil. Additionally, certain species have been isolated from samples of the

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epidermis or vagina (Mccurdy and Morrow, 2003). Nowadays, a greater number of Cryptococcus spp. are known as human pathogens; these species are classified as emerging yeast pathogens, and effective treatment strategies for them remain elusive (Khawcharoenporn, et al., 2007).

Microbial biofilms consist of communities of surface-attached microorganisms (sessile cells) that are intricately organized and encapsulated in an extracellular polymeric matrix (EPM) that they generate themselves. Biofilms afford microorganisms a multitude of benefits, including enhanced cell-to-cell interactions and protection from environmental stresses (e.g., fluctuations in pH, temperature, and nutrient availability) and predation (Ravi, et al., 2009). The biofilm growth pattern is primarily observed in natural environments and serves as a significant mechanism for the pathogenicity of diverse yeast in animal and plant hosts (Donlan and Costerton, 2002). The study aims to identify Cryptococcus spp. in Erbil city in the Kurdistan region of Iraq by phenotyping and molecular methods and confirm it by several tests.

II. MATERIAL AND METHODS

A. Samples Collection

Two hundred samples of pigeon droppings were collected from 53 different regions in Erbil city and its suburbs from November 2021 to October 2022. Samples have been collected and transferred in sterilized and sealed containers into the advanced mycology laboratory at Science College/ Salahaddin University.

Every sample has been processed and homogenized in a biosafety cabinet. After five minutes of vortexing and five minutes of centrifuging at 500Xg, then 20 grams of Pigeon (Columbia Livia) dropping samples were suspended in sterile phosphate-buffered saline (PBS) at a ratio of 1:5. From each tube's 100 µL of supernatant was used to inoculate Niger Bird Seed Agar (NBSA) in a Petri dish and Sabouraud dextrose agar (SDA) plates with 40 mL of chloramphenicol (50 µg /mL) and 1.0 g creatinine. For three to ten days, the petri dishes were incubated in the dark at 37 °C under humid circumstances. (Edberg, et al., 1980; Oh and Hwang, 2005; Sirag, et al., 2021). Positive yeast colonies were subcultured on SDA slants for further identification tests and preservation.

B. Phenotypic method of yeast

Fifteen Cryptococcus isolates have been obtained from pigeon droppings in different places around the Erbil Suburban. After 48 hours of incubation at 37 ºC, each isolate was recognized by culturing on SDA and NBSA to identify the suspected colonies. All positive cultures were then identified microscopically by staining with negative stain India ink and evaluated at a magnification of 40X.

C. Bio typing method

Using VITEK, the urease test, and the phenol oxidase test, biochemical techniques were used to identify the isolated yeast.

1. VITEK test for identification

To transfer a suitable number of colonies of a pure culture of yeast into a 12 × 75 mm clear plastic (polystyrene) test tube, a sterile swab or applicator stick has been used, then 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) has been added. Hence, the turbidity is modified and measured using a turbidity meter called the DensiChek™ as tabulated in table-1.

TABEL I
SUPENSION TURBIDITIES USED FOR CARD INOCULATION

Product	McFarland Turbidity Range
GN	0.50-0.63
GP	0.50-0.63
YES	1.80-2.20
BCL	1.80-2.20

GN: Gram-negative, GP: Gram-positive, YES: Yeast, BCL: Bacterial cell

2. Urea test examination

Using a urea agar slant, the urease test was performed to see if the colour changed from yellow to pink following inoculation and incubation. All positive

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tested isolates were recognized as Cryptococcus species. (Kadhim, 2023).

3. Phenol oxidase test

Assays for phenol oxidase were among the biochemical tests used to further establish the purity of the colonies. Colonies were rubbed on paper soaked in NNN'N'-tetramethyl-p-phenylenediamine for the oxidase test, and the colour of the colonies was checked for bluish-purple appearance. (Kadhim, 2023).

4. Molecular method

DNA extraction and PCR amplification

Using a genomic DNA extraction kit (Fungi/Yeast Genomic DNA isolation kit. Jena, Bioscience/Germany), DNA was extracted from the yeast isolates. The yeast isolates were cultured on SDA for 48 hours at 37°C and the protocol was used to extract DNA as tabulated in table-2.

TABEL II
THE KIT COMPONENTS

Buffers
Cell Lysis Solution
Cell Resuspension Solution
Proteinase K (20 mg/ml)
Protein Precipitation Solution
Washing Buffer
DNA Hydration Solution
Rnase A (4 mg/ml)

The Sequence Alignment for Targeted Region:

The sequences were analyzed using MEGA5 and NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). PCR results were sequenced on an Applied Biosystems 3500 Genetic Analyzer. The sequencing data was subsequently submitted to the National Centre for Biotechnology Information (NCBI) for nucleotide sequence accession numbers.

PCR Amplification of ITS region sequences

Using universal primers developed by (White, et. al., 1990).) the target sequence 500-600 bp of the Cryptococcus sp. in the rDNA fragments was successfully amplified. A total of 25 µl of PCR master mix reaction volume was used, which included 3 µl of genomic DNA, 12.5 µl of 2X GoTaqGreen Master Mix (Promega/USA), and 1 µl for each forward and reverse primer for both ITS1 and ITS4 primer as shown in table-3. The mixture was then finished by adding 7.5 µl of nuclease-free water. The PCR amplification was performed on a Techne/UK thermocycler under the following conditions: an initial denaturation cycle at 95 °C for 5 minutes, followed by 35 cycles at 94 °C for 1 minute, 55 °C for 1 minute, 72 °C for 1 minute, and 72 °C for 7 minutes. The size of the PCR products was validated using 2% agarose gel electrophoresis in 1X of Tris/Borate/EDTA(TBE) buffer, and the PCR products from Cryptococcus isolates were sent to Macrogen in South Korea for sequencing.

TABEL III
THE SEQUENCE OF UNIVERSAL PRIMERS

Gene-Region	The Sequence of Primer
ITS1	5-TCC GTA GGT GAA CCT GCG G- '3
ITS4	5' TCC TCC GCT TAT TGA TAT GC- '3

Biofilm formation

Congo Red Method (CRA)

It is an alternate strategy for the development of biofilms described by (Freeman et al., 1989). Success was indicated by black colonies with a dry crystalline quality. A non-biofilm manufacturer does not usually change their colour. The experiments were done in three independent runs, each in duplicate.

Microtiter Plate Method (MTP)

The microtiter plate assay described by (Millsap, et al.,

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2001) is the most widely used approach and has long been considered as a gold standard for detecting biofilm growth. The current study looked at the ability of Cryptococcus isolates to produce biofilm. For biofilm development, a sterile, flat-bottomed 96-well microtiter plate was utilized. Sabouraud dextrose broth (SDB) is made with powdered Sabouraud broth (HiMedia, Mumbai, India) and 60 grams of glucose per liter. Fresh, pure cultures of testing Cryptococcus isolates were injected for 24 hours at 37 °C in a modified SDB (8 percent glucose content) (Ramage, et al., 2001). Biofilm development was discovered by adding 100 µl of this standardized cell suspension to microtiter plate wells that previously contained 100 µl of fresh SDB medium, and only broth served as a control to assess sterility and non-specific media binding. The microtiter plates were incubated for 48-72 hours at 37 °C. Following incubation, the contents of each well were tapped out and washed four times with 200 µl of phosphate-buffered saline (pH 7.2) to eliminate free-floating planktonic cells.The biofilms were stained with 1 percent crystal violet in microplates containing adherent (sessile) cells. The extra stain was properly wiped off with deionized water after 20 minutes, and the plates were allowed to dry. Cryptococcus cells adhered to the wells were equally stained with crystal violet and typically formed a biofilm at the bottom of the wells. (Martinez and Casadevall, 2007).

ALS1 and HWP1 Genes Detection which are Linked to Biofilm Development

In the multiplex PCR amplification, ALS1 and HWP1 were utilized as shown in table-4. A total of 25 µl of PCR master mix reaction volume, including 3 µl of genomic DNA, was used. 12.5 µl of 2X GoTaqGreen Master Mix (Promega/USA) and 1 µl for each of the forward and reverse primers for both genes were added, and the volume was finished with 5.5 µl of DNase, RNase-free water. For the PCR, the following methodology was used: 1 cycle at 94 degrees Celsius for 4 minutes, followed by 35 cycles at 94 degrees Celsius for 30 seconds, 52 degrees Celsius for 1 minute, and 72 degrees Celsius for 2 minutes. At 72 °C, a 5-minute final extension cycle was performed. The successful amplification was done after the PCR products were separated on agarose (2%), 318 bp, and 572 bp for both ALS1 and HWP1 genes (Wealtec, Dolphin-View, USA) (İNCİ, et. al., 2013).

TABEL IV
THE SEQUENCE OF BIOFILM FORMATION PRIMERS (İNCİ, et. al., 2013)

Gene	The Sequence of Primer
ALS1	F5' GACTAGTGAACCAACAAATACCAGA-'3
ALS1	R 5' -CCAGAAGAAACAGCAGGTGA-'3
HWP1	F 5' -ATGACTCCAGCTGGTTC-'3
HWP1	R 5' -TAGATCAAGAATGCAGC-'3

III. RESULTS AND DISCUSSION

Among 200 pigeon droppings samples collected from 53 positions in Azadi, Badawa, Bahare kon, Bahare nwee, Barzan, Berkote nwee, Bnaslawa, Chnar, Chwar chra, Daratu, Darwazae shar, Dawajen, Farmanbaran, Galawezh, 121 Gulan, Hakmawa, 8Hasarok, 5Hasarok, Havalan, Kany gany, Kasnazan, Karezan, Kotr city, Kuran, Mahabad, Mahala arab, Makhmur, Mala omer, Mamostayan, Mantkawa, Masif Salahaddin, Mufti, Muhandsen, Nawroz, Qarabu, Ronaky, Roshenbere, Saydawa, Science college, Setaqan, Shadi, Share zaytun, Shawes, Shorsh, Shurtawa, Swere gchka, Tajeel, Tayrawa, Toraq, Xanaqa, Zanko, Zanko village and Zhean in Erbil city- Iraq, Cryptococcus laurentii, many non-cryptococcal yeasts, molds and other cryptococcus species have been identified. All isolates phenotypically were identified microscopically. Cryptococcus laurentii was identified on SDA and NBSA following a 48-hour incubation period at 37 ºC based on capsule formation as shown in Figs. 1 and 2. Using SDA and NBSA to isolate Cryptococcus laurentii, which produces creamy colonies on both media, is considered a limited resource. Similar findings were reported elsewhere in dairy cows (Kadhim, 2023).

Pathogenic species such as C. laurentii and C. luteolus, isolation and identification highlight the relevance of biodiversity and identifying pathogenic species' environmental niches because these species can

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enhance the risk of infection in immunocompromised people (Teodoro, et al., 2013).

In 2002, Averbuch et al. isolated and identified C. laurentii from the blood of a patient diagnosed with aganglioneuroblastoma. This study is very important because it demonstrated the presence of this species in a patient with cancer and determined that the isolate was resistant to fluconazole (Averbuch, et al., 2002). In opposite to our results a study in Pelotas, State of Rio Grande do Sul, isolated samples from sources with large amounts of droppings, such as church spires, rice mills, warehouses, parks, historic buildings, and outdoor locations. However, only one sample tested was positive for C. neoformans (De Faria, et al., 2010).

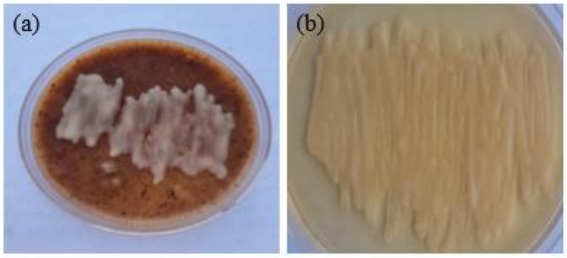

Fig. 1. Cryptococcus laurentii on (a) NBSA and (b) SDA.

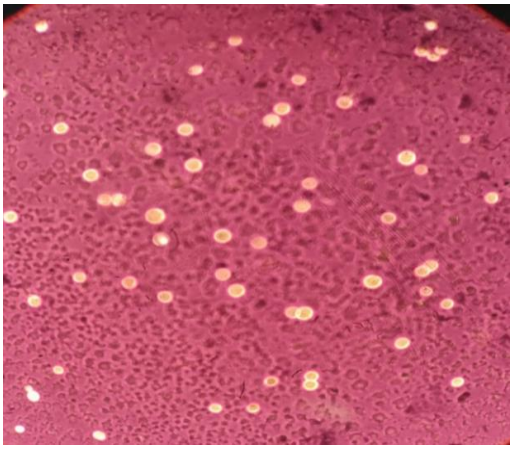

Fig. 2. Cryptococcus laurentii capsule with India ink-negative stain.

The Vitek MS system (bioMérieux, Marcy l'Etoile, France) is a relatively new commercialized MALDI-TOF-based method and is optimized for both bacterial and fungal identification utilizing single-spot inoculations without prior protein extraction. Several studies have evaluated the performance of the Vitek MS system with database version 1.0 in yeast identification (Hata, et al., 2007). The result of biotyping using the VITEK test indicated C.laurentii in our findings does not agree with those found by others (Hata, et al., 2007). When species of Cryptococcus were tested, the YST card correctly identified 33/36 isolates. One isolate of each of Cryptococcus neoformans and Cryptococcus uniguttulatus was incorrectly identified as Rhodotorula glutinis/Rhodotorula mucilaginosa/Cryptococcus laurentii by YST. One isolate of Cryptococcus laurentii was unidentified by the YST card (Hata, et al., 2007).

It has been observed that all strains of Cryptococcus neoformans, including the variation Cryptococcus uniguttulatus (Zach), were capable of rapidly hydrolyzing urea, regardless of whether they came from human or animal sources or were isolated from soil specimens (Seeliger, 1956). Furthermore, a positive reaction was also obtained with C. laurentii which was found to be related serologically to C. neoformans (Seeliger, 1956).

Our results agree with what has been found by another research group (McTaggart, et al., 2011). When evaluated using Remel rapid urea broth and urease test broth, isolates of Cryptococcus, Trichosporon, and Rhodotorula were found to turn the medium bright pink, while other species of yeast produced a negative reaction (McTaggart, et al., 2011) as shown in Fig. 3.

Fig. 3. Urease test of Cryptococcus Laurentii: Yellow is control and Pink is positive.

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Early studies of phenol oxidase activity of Cryptococcus sp. focused on whole-cell assays relying on the detection of visible pigment. Staib was the first who describe this pigment to be unique to Cryptococcus neoformans, requiring a substrate found in Guizotia abysinica extracts (Staib, 1962). Our results for Cryptococcus laurentii indicated that it has the efficiency for phenol oxidase positive test. It was found that a given strain produces different phenol oxidases under different conditions. also, the activity of phenol oxidase in 37 °C cultures is lower than that of phenol oxidase in 25 °C cultures as shown in Fig. 4.

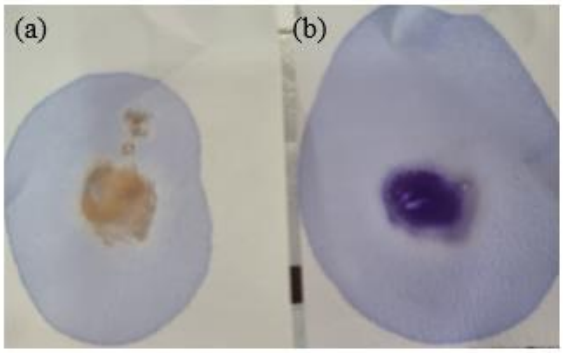

Fig. 4. Phenol oxidase test of Cryptococcus laurentii A-negative B- positive right

The results of the molecular identification by using universal gene amplification were consistent with the phenotypic analysis which included Cryptococcus laurentii. Molecular data of specimens from ITS nucleotide sequences provided accurate characterization and identification of isolates (See Fig. 5). The percentage of the alignment nucleotide sequences was arranged between 99.8 and 100%. The sequence data of Cryptococcus laurentii provided GenBank accession numbers for nucleotide sequences as tabulated in table-5.

TABEL V
GENBANK ACCESSION NUMBER FOR *C. LAURENTII* ISOLATE

Cryptococcus Laurentii	GenBank Accession Number
1	OR509919

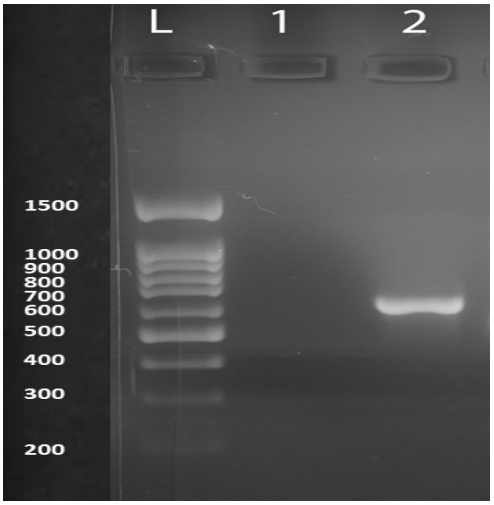

Fig. 5. PCR amplification of Internal transcribed spacer. Lane L: Ladder 100bp, Lane 2: nuclease-free water as a negative control, Lane 3: Correspond to Fungal (ITS) region(600bp).

Multiplex PCR permits the amplification of two or more loci in a single reaction and can be done fast with less DNA. As a result, it is yet another promising approach for microbiological diagnostics (Edwards, et al., 1994). The utilization of this method in the investigation of cryptococcosis has predominantly concerned itself with the detection of C. neoformans and C. gattii, including their serotypes, through the implementation of various primer combinations that enhance specificity and sensitivity, or in conjunction with other molecular methodologies, such as real-time PCR (Ito-Kuwa, et al., 2007). Additionally, multiplex PCR has also been used to determine the mating-type profile of fungal isolates (Esposta, et al., 2004).

Nevertheless, strains of Cryptococcus that are not classified as neoformans or gattii Cryptococcus species have been identified in cases of cryptococcosis among both immunosuppressed and immunocompetent individuals in recent times. The species C. curvatus and C. laurentii were identified using the polymerase chain reaction (PCR) technique (Nowicka, et al., 2007).

However, infections in the bloodstream and diseases in neuromeningeal, pulmonary, cutaneous, and peritoneal have been associated with C.laurentii

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(Smith, et al., 2017).
A similar prevalence of non-neoformans and non-gattii Cryptococcus infections were reported in three states of the United States of America (USA) in 2020 and during the same study, C. laurentii, C. liquefaciens, and C. magnus were considered as pathogenic (Cano, et al., 2020).

Fungi capable of forming biofilms are commonly obtained from indwelling biomedical equipment and have been linked to many human disorders (Ramage, et al., 2009). The utilization of scanning electron microscopy (SEM) allowed for the examination of C. laurentii biofilms, revealing surface topographies characterized by abundant growth and dense colonization. Furthermore, the presence of extensive polymeric substances surrounding the cells was observed (Ajesh and Sreejith, 2012).
In the biofilm creation study, both phenotypic (using CRA and Micro Plate Method) and molecular (using PCR to detect virulence genes like ALS1 and HWP1) approaches yielded biofilm producers. Gene presence and phenotypic correlation during biofilm formation were studied. The results showed that the C.laurentii isolate possessed both the ALS1 and HWP1 genes as shown in Fig. 6.

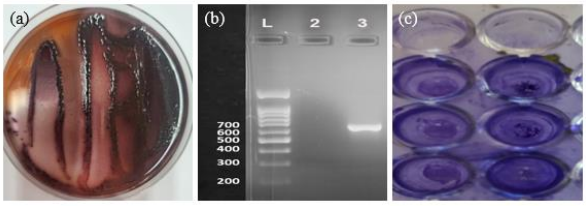

Fig. 6. Biofilm formation by (a) Congo red agar (CRA), (b) PCR amplification of HWP1 gene. Lane L: Ladder 100 bp, Lane 2: nuclease-free water as a negative control, Lane 3: Correspond to HWP1 gene (642 bp) and (c) Microtiter plate.

Interestingly, it has been reported that both C. neoformans and C. laurentii shared a similar pattern of biofilm growth reaching maturation at 48 h with no significant difference in the metabolic activity rates up to 72 hrs (Kumari, et al., 2017). Moreover, it has shown that 60% of Cry. laurentii strains showed low biofilm formation, and the other 40% were considered to be moderate biofilm producers (Oliveira, et al., 2021).

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