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Isolation and molecular identification of Pathogenic cryptococcus gattii from natural habitat

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ABSTRACT

This study is designed to isolate and molecular identification of C. gattii, C. gattii is pathogenic yeast and effect immunocomposed and immunocompetent, Methods: collect 50 samples from eucalyptus leaves. The collection time was extended from November 2021 to February 2022 and then culture at SDA, Cryptococcus Differential Agar esculin agar and Eucalyptus leaves agar, Brain heart infusion agar with methyldopa and Brain heart infusion agar with methyldopa media, biochemical test including urease test, and then confirm identification by molecular identification by PCR technique sequencing and genetic analysis. The results showed that 4 swaps taken from eucalyptus leaves included cryptococcus neoformans. This study indicated that the virulence and normal habitat of cryptococcus gattii. These results indicate that there may be a risk for human exposure to cryptococcal disease due to inhalation of basidiospores from eucalyptus leaves.

1. Introduction

Over many years, there have been many scientific studies to describe fungi species and their behaviors. however, with the total number of 1.5 million species that have been estimated, only 80 000 -120 000 fungi species have been identified so far. (Hawksworth, 2001; Kirk et al., 2001). The principle fungi causing the mycosis are responsible for (1) superficial and cutaneous mycosis (2) subcutaneous mycosis (3) deep systemic mycosis caused by the opportunistic yeasts or filamentous fungi. Infections with Cryptococcosis, Candidiasis, and Aspergillosis have been documented in the United States, Europe, Africa, and other nations. (De Hoog et al., 2005).

In suitable environments, the Cryptococcus fungus can infect both humans and animals, causing a disease known as cryptococcosis. People who have HIV (Human Immunodeficiency Virus) infection or who are otherwise immunocompromised are more susceptible to getting the disease. The infection could affect the lungs, the central nervous system, or other organs. Cryptococcus is a genus with around 100 species, C. gattii and C.neoformans are two Cryptococcus species that are usually related with infections in humans. The fungus is widespread throughout the world, particularly in tropical and subtropical regions, as well as in temperate regions. although, cryptococcal disease infects different patient populations the main mechanism that is involved in the causation is Immune suppression. Diabetes, AIDS, , chronic liver disease, prolonged use of steroids, chronic renal disease, and organ transplant patients are all related to the development of cryptococcal disease. (Lin, et al., 2015).

C. gattii is an asexual budding yeast that may be found living in the environment, as well as in people and other animals. It is a basidiomycetous yeast. The process of reproduction may take place between cells of different mating types or between cells of the same mating type (Fraser et al., 2005). Yeast cells go through a metamorphosis known as the transition to hyphal growth during the sexual phase of development. This results in the formation of mycelium and basidiospores (Kwon-Chung et al., 1978). It is not yet known whether the infectious propagules are dehydrated yeast cells, blastospores that were formed sexually, or basidiospores that were produced sexually. Since a long time ago, C. gattii has been acknowledged as an endemic pathogen in Australia. During the decade of the 1990s. Major advancements in molecular technology have led to a reevaluation of the taxonomy and phylogeny of this species, as well as a better understanding of the connections between its ecology, epidemiology, and clinical (Chen et al., 2014).

2. Methodology

Baghdad was the location where a total of 25 samples of eucalyptus leaves were gathered and placed in sterile plastic bags (Talbieh area and Palestine Street area). After being placed on ice and transported to the Zoonotic Diseases Unite at the College of Veterinary Medicine, the samples that were obtained were placed in clean, sterile plastic bags. In order to get the samples ready for analysis, they were first washed in sterile distilled water, then treated in sterile saline solution that was augmented with chloramphenicol (10.0 mg/mL), and finally homogenized using ultra-homogenization for four minutes. All of these steps were done in order to get the samples ready for analysis. After that, the bottle was let to sit at room temperature for half an hour to allow the sediment to settle.

After the samples were homogenized, a loopful of each was scattered onto plates of Sabouraud dextrose agar with chloramphenicol, and the plates were incubated at 30 degrees Celsius for 48 hours under normal environmental conditions. If the growth appeared to be diagnosed by doing a smear on a slide and staining it with lactophenol cotton blue in addition to the urease test, and if it was confirmed by culturing on the cryptococcus differential agar media and other identification tests, then it was determined that the growth was cryptococcal in nature (**Elhariri *et al*., 2016).** The period for collecting was moved forward to March 2022, from its original date of November 2021.

Molecular Characterization

The extraction of DNA

The DNA was extracted using pure fungal cultures, and the isolates were inoculated using 1.5 ml Eppendorf tubes containing 0.5 ml of Sabouraud Dextrose broth supplemented with chloramphenicol. The tubes were then placed in an orbital shaker at 150 rpm at 30°C for a period of one night.

After 24 hours, the predetermined concentration suspensions were centrifuged for ten minutes at 5,000 rpm and the pellet was frosted for an hour at -20 degrees Celsius. The pellet was then incubated for an additional hour at 65 degrees Celsius in 0.5 milliliters of extraction buffer that contained 50 millimoles of Tris-HCl, 50 millimoles of EDTA, three percent sodium dodecyl sulfate, and one milliliter of 2-mercap To extract the lysate, a mixture of phenol, chloroform, and isoamyl alcohol with a volume-to-volume (v/v) ratio of 25:24:1 was used. After that, 75 µl of 1M sodium chloride and 65 µl of 3M sodium acetate were combined, and the resulting volume was incubated at 4 degrees Celsius for half an hour. In the end, an isopropanol precipitation was employed to extract the DNA, which was then washed with an ethanol solution that was composed of 70 percent (v/v) alcohol. The concentration of DNA was measured using a UV-VIS Spectrophotometer at 260 nm, and then it was stored at -20 degrees Celsius until future usage. (**Tendulkar et al., 2003; Mseddi et al., 2011; Moťková, & Vytřasová, 2011**).

Amplification of PCR

The D1/D2 sections of the primers that were utilized for amplification targeted ITS1 and ITS4 for the purpose of identifying different species of Cryptococcus. The length of the expected fragments ranges from 500 to 650 base pairs. Information on the primers may be found in Table 1. The amplification procedure began with one cycle of initial denaturation performed at 940 degrees Celsius for thirty minutes. This was followed by 35 cycles of denaturation performed for one minute at 940 degrees Celsius, primer annealing performed for one minute at 550 degrees Celsius, chain extension performed for one minute at 720 degrees Celsius, and a final extension performed for seven minutes at 720 degrees Celsius. After the PCR products were amplified, they were separated using an agarose gel with a concentration of 1.5 percent and visualized using a UV gel documentation system. After that, the amplicons were stored at a temperature of -200 degrees Celsius so that they could be examined further. (**Mitchell et al., 1994; Lau et al., 2007; Lusia Leal et al., 2008; Sidrim et al., 2010**).

The primers used in the interaction

Table 1 The specific primer of gene ITS4

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5′- TCCGTAGGTGAACCTGCGG -3′	60.3	50 %	500-650 base pair
Reverse	5′ TCCTCCGCTTATTGATATGC-3′	57.8	41 %	

3. Results and Discussion

According to the primary method of isolation, which relied on (direct examination) characterization of Cryptococcus gattii growth on SDA, cryptococcus differential agar, Brain heart infusion agar with methyldopa, urease test, and Eucalyptus leaves agar media, the organism was able to survive and grow. 4 (4 percent) eucalyptus leaves samples out of 25 isolations.

Table 2 Primary isolation in lab

Sources of sample	No. of samples	Primary isolation in lab	
		No. of +ve	%
Eucalyptus leaves	50	7	14%

After just two to three days of incubation, the yeast will have formed colonies on the Sabouraud dextrose agar that are white, creamy, convex, and shiny. The mucoid appearance of colonies formed by C. gattii is due to the development of a polysaccharide capsule by the cells; these findings were in accordance with what was previously known (**Khanal et al., 2002**).

Cryptococcus Differential Agar

The development and production of pigment in almost every strain of C. gattii need glucose as a necessary component. C. gaitii has the ability to absorb D, but C. neoformans does not. -tryptophan (**Baró *et al.*, 1998**), which results in the production of a brown pigment that may diffuse (**Chang *et al*., 2015**). Glycine is the only source of carbon and nitrogen that Cryptococcus gaitti can consume in its metabolic processes (**Acevedo Almendarez, 2015**).

Figure 1 C. gatti appear as Brown, mucoid colony like this photo.

Brain heart infusion agar with methyldopa

When cultivated in agar media, colonies of melanin-producing Cryptococcus species display a rainbow of hues ranging from brown to black. Methyldopa infusion agar for the brain and heart (**Menezes *et al*., 2011**).

Figure 2 C. gattii in agar media Brain heart infusion agar with methyldopa

Eucalyptus leaves agar media

Colonies of melanin-producing Cryptococcus species show a display of colors varying from brown. Control and brown pigmented colonies of positive control and environmental isolates. Brown color effect of C. neoformans on Eucalyptus leaves agar media I (**Elhariri *et al*., 2016**).

Figure 3 C. gattii appear brown

Urease test

After 48 hours of incubation at 30-33 C, the isolated yeast strains displayed the ability to use urea and liberate ammonia by generating urease enzyme and altering the color of the medium from yellow to pink or light purple, which is compatible with the findings of (**Li & Wu, 1992**).

Figure 4Control urease test Positive urease test for cryptococcus gattii.

Table 3 Results of sequencing and genetic analysis

Sources of sample	No. of samples	Results of sequencing and genetic analysis	
		No. of +ve	%
Eucalyptus leaves	**7**	**4**	**57.14%**

After executing an NCBI blastn for each of their PCR amplicons, the sequencing reactions provided conclusive evidence about the true identities of these infectious sequences (**Zhang *et al*., 2000**). The BLASTn engine at NCBI discovered that 99 percent of the 500-650 bp amplicons had a sequence that was comparable to either the targeted reference target sequences or the sequences of the sequenced samples. The specific locations and other characteristics of the obtained PCR fragments were discovered by comparing the observed nucleic acid sequences of these studied samples with the retrieved nucleic acid sequences. This allowed for the identification of the exact placements of the retrieved PCR fragments (MH043591.1). The NCBI server was used to calculate the total length of the specified target gene. Additionally, the start and end locations of the targeted gene were verified with the help of the most similar fungal target. After inserting the 500-650 bp amplicons within the genomic sequences of the fungus, the properties of the ITS4 sequences were underlined, and the total length of the amplified fragments was also assessed.

The location and size of the PCR amplicons that range from 500 to 650 base pairs and are utilized to amplify ribosomal sequences from within the genomic sequences of Cryptococcus gattii (GenBank acc. no. MH043591.1).

When compared to the most similar referencing reference nucleic acid sequence, the alignment findings of the 500 650 bp samples reveal an interesting nucleic acid variation: the occurrence of two nucleic acid variants, each of which is distinguished by a number of nucleic acid changes. It was discovered that these nucleic acid alterations were distributed in a very particular manner among the studied fungus samples. The sequencing results showed that mluti variants, T-A 148, G-C 283 and C-T 430.

The T-A 148, G-C 283 and C-T 430. Verification and documentation were carried out on the very high variation confirmation, sequencing chromatograms of the samples that were examined, and the thorough annotations of those samples. The chromatograms of their respective sequences were shown in the order in which their locations appeared in the PCR amplicons. The existence of each variation was verified in the chromatogram that was used to create it, as was the lack of any kind of technical mistake that may have occurred.

To provide a phylogenetic understanding of the actual distances between the current investigated samples and their relative sequences, a comprehensive phylogenetic tree was constructed in the current study based on nucleic acid variations observed in the amplified 500-650bp ribosomal sequences amplicons. This tree was constructed in order to provide a phylogenetic understanding of the relationships between the investigated samples.

This phylogenetic tree comprised not only the sample that was being studied at the time but also additional nucleic acid sequences that were related to C. gattii.

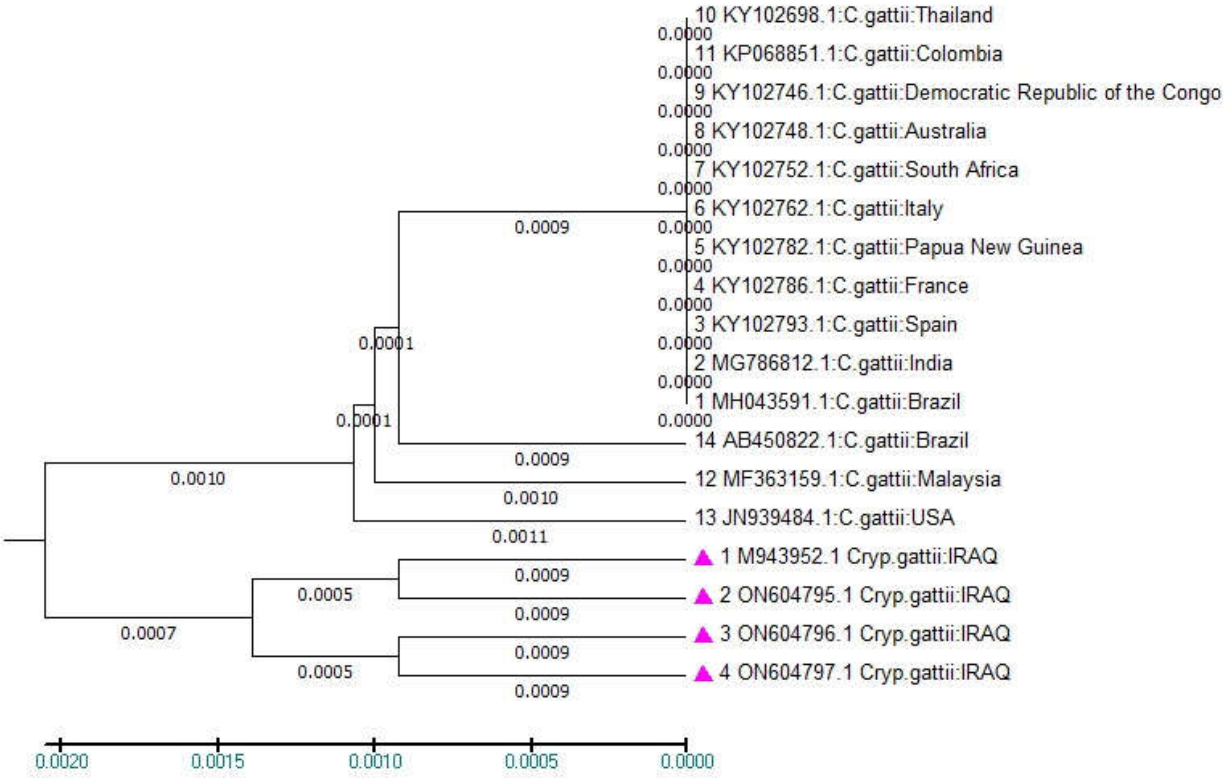

Figure 5 The phylogenetic tree have 14

Because it was discovered that GenBank isolates with the accession numbers AB450822.1, JN939484.1, and MF363159.1 which was deposited from Brazil, the United States of America, and Malaysia matching is get 99 percent, it was determined that the observations made through the phylogenetic tree regarding my isolate were confirmed by other neighboring sequences.

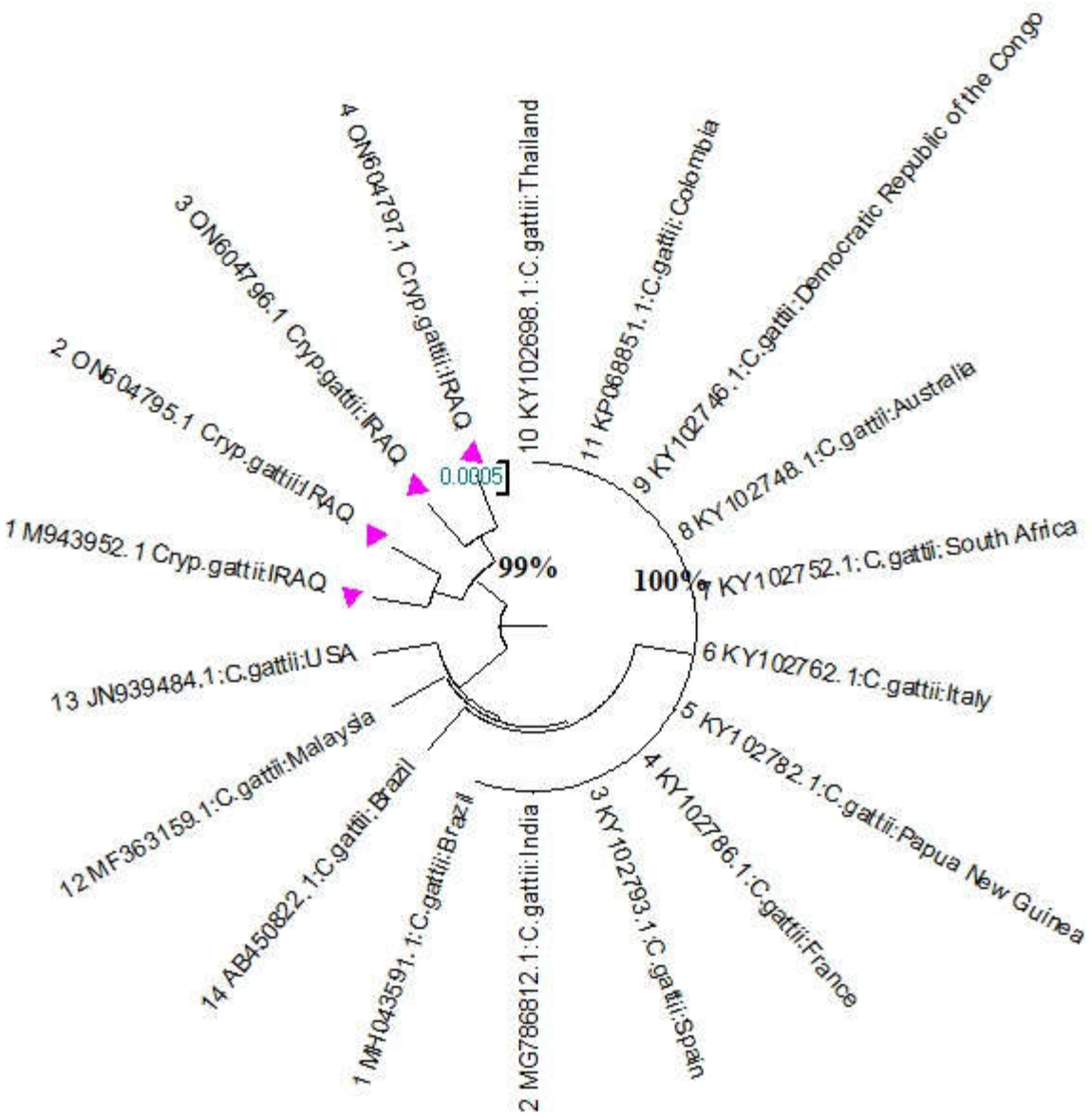

Figure 6 Evolutionary analysis by Maximum Likelihood method

For the purpose of deducing the evolutionary history, both the Tamura-Nei model and the Maximum Likelihood technique were used (**Tamura & Nei, 1993**). A focus is placed on the tree that has the highest log probability (-261.47). Next to the branches is a representation of the proportion of trees in which the relevant taxonomic groups grouped together. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with the superior log likelihood value. This resulted in the formation of the initial tree(s) for the heuristic search. Next to each internal node in the tree is an indication of the percentage of sites within that descending clade that have at least one place with an unambiguous base in at least one sequence. In this particular investigation, seven different nucleotide sequences were used. The final dataset had a total of 149 different places in its entirety. In order to carry out evolutionary analysis, MEGA 6 was used (**Tamura *et al*., 2013**).

4. Conclusion

According to the results obtained in this research, c. gattii approved to isolate from eucalyptus trees in about 7 in percent of 14% but this results when conformed by PCR analysis and DNA sequencing restricted in four isolate with 57.14%, so that isolation of such fastidious pathogenic yeast required accurate, sensitive and specific technique more than that use in academic laboratories to conformed isolation and identification to such pathogenic yeast.

Even through, the primary isolation of other cryptococcus species with it should get a lot of attention, because that cryptococcus species were approved to be highly pathogenic for both immunocompromised and immunocompetent individuals.

Be aware of the areas where eucalyptus trees has been grown especially in case of immunocompromised individuals and this is done by using mask and gloves.

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