RESEARCH ARTICLE

Multiplex PCR - RFLP Assay for Identification of Cryptococcus neoformans and Cryptococcus gattii Isolated from Birds’ Droppings and Eucalyptus Trees

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Abstract

Cryptococcus neoformans/ Cryptococcus gattii (C. neoformans/ C. gattii) species complex are encapsulated basidiomycetous yeasts, causing cryptococcosis which is a life-threatening fungal disease of the pulmonary and central nervous system of humans and animals. This study aimed to investigate the recovery rate of C. neoformans and C. gattii from bird droppings and Eucalyptus trees in Egypt as well as the performance of the phenotypic and molecular identification methods for Cryptococcus species identification. Overall, 27 Cryptococcus isolates (13.5%) were isolated from 200 examined samples including 70 pigeon droppings, 50 captive birds’ droppings, and 80 Eucalyptus trees samples. The recovered isolates were phenotypically identified based on macro- and micro-morphological characters, urease test, and differentiation using cryptococcus differential agar media. Multiplex polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis using EcoRI restriction enzyme for confirmation of species identification. The molecular methods identified 20 C. neoformans from pigeon’s droppings (12/70, 17.14%) and captive birds’ droppings samples (8/50, 16%), as well as 7 C. gattii from Eucalyptus trees (7/80, 8.75%). Molecular identification results did not correspond with those of the phenotypic identification methods in three isolates (11.11%), as phenotypic methods identified only 4 C. gattii isolates and molecular methods identified 7 isolates. In conclusion, multiplex PCR and RFLP analysis of multiplex PCR products are rapid, sensitive, species-specific, and more reliable methods for identification of Cryptococcus species and may be used as a complementary to phenotypic methods to avoid false-negative and false-positive results.

Key words: C. neoformans, C. gattii, Identification, RFLP assay, Multiplex PCR.

Introduction

Cryptococcosis is a potentially invasive fungal infectious disease of the pulmonary and central nervous system of humans and animals. It affects immunocompromised hosts, causing an estimated one million new cases and over 625,000 deaths per year globally [1, 2]. This disease is mainly caused by encapsulated basidiomycetous yeasts C. neoformans/C. gattii species complex [3, 4]. Cryptococcosis is also very important in a wide range of animals worldwide as cats, dogs, horses, cattle, sheep, goats, and birds. It is commonly associated with mastitis in cattle, sheep, and goats in addition to endometritis and placentitis in mares [5]. Cryptococcus infections are rare in birds [6]. Although C. neoformans and C. gattii are ubiquitous yeasts, their main ecological niches are relatively different [7]. Cryptococcus neoformans is worldwide distributing in the environment and is
associated with bird excreta, especially pigeon droppings and soil [8]. While C. gattii can be associated with soil debris and different tree species especially Eucalyptus camaldulensis and is limited to tropical and subtropical areas [9].

Traditionally C. neoformans and C. gattii have been identified by conventional and phenotypic methods as capsule detection by India ink stain, phenoloxidase test focusing on melanin production, and growth at 37°C [10]. The differentiation between the two species can be performed based on the potential of using glycine as a source of carbon and nitrogen, as well as the resistance or sensitivity to canavanine [11].

Various molecular techniques have been employed for the identification and genotyping of C. neoformans and C. gattii. They have great sensitivity and specificity, with the ability to overcome the false positive and false negative and time consuming limitations of the conventional phenotypic methods [8, 12]. Different techniques were applied in identification as DNA hybridization, polymerase chain reaction (PCR) with specific primers [13]. However, genotyping, epidemiology, and genetic diversity of Cryptococcus species have been investigated by using numerous molecular methods including PCR fingerprinting, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST), multilocus microsatellite typing (MLMT), and whole genome sequencing [14, 8, 15]. Recently, other new techniques have been developed including Luminex xMAP, hyper-branched rolling circle amplification (HRCA), as well as matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS), and it might have a great opportunity in laboratory diagnosis [16-18].

Available studies conducted in Egypt have performed PCR amplification of capsular genes CAP 64 [19, 20], CAP 10, and CAP 60 [21] for identification of environmental Cryptococcus species. Thus, this research was carried out to evaluate multiplex PCR-RFLP assays for identification of C. neoformans/ C. gattii species complex from environmental sources.

This study was designed to shed light on the frequency of C. neoformans and C. gattii from birds’ droppings and Eucalyptus trees as well as the diagnostic performance comparison of phenotypic and molecular identification methods.

**Materials and methods**

**Samples collection**

A total of 200 environmental samples were randomly collected from different Governorates in Egypt. These samples represented 70 pigeon droppings and 50 captive birds’ droppings collected from pet shops, houses, and towers in Sharkia, Dakahlia, and Qalubia Governorates. In addition, 80 Eucalyptus trees samples taken from different areas in Sharkia Governorate and Cairo agriculture road. The collected samples were placed in sterile plastic bags and transferred to the laboratory for mycological examination.

**Isolation of Cryptococcus species**

One gram of each bird dropping sample was suspended in 9 ml sterile saline solution, mixed, and centrifuged at 3000 rpm for 5 min [22]. The supernatant was discarded and the sediment was obtained. Five grams of each Eucalyptus tree sample was suspended in 25 ml sterile saline solution, vortexed, and allowed to settle down for about 20 min. [23]. After that, a loopful from each sample was streaked on two Petri dishes of Saboraud dextrose agar (SDA) with chloramphenicol (5 µg/ml) (Himedia, India) and then incubated at 25°C and 37°C for 72 h.

Creamy, tan, and brown mucoid yeast colonies were picked up by sterile loop from primary culture onto SDA slopes.

**Phenotypic Identification**

Phenotypic identification of cryptococcal isolates was done using standard methods including microscopy with Gram’s stain and India ink, ability to grow at 37 °C, and biochemical test for urease activity [24, 25]. Finally, cryptococcus
differential agar media (HiMedia, India) was used for differentiation of \textit{C. neoformans} and \textit{C. gattii} on the basis of assimilation of tryptophan and glycine as carbon and nitrogen sources.

\textbf{Molecular identification}

DNA was extracted using QIAamp DNA mini kit (Catalogue No. 51304, Sigma, USA) according to manufacturer’s instructions.

\textbf{Multiplex PCR}

PCR amplification was performed in T3 Thermal cycler (Biometra, Germany) in a final volume of 50 μL per sample consisting of 25 μL of Emerald Amp GT PCR master-mix (Code No. RR310A Takara, USA) (2x premix), 1 μL (20 pmol concentration) from forward and reverse primers, 6 μL of template DNA, and nuclease-free water up to 50 μL. Oligonucleotide primers were CNa-70S 5’ ATTGCGTCCAAGGAGGCTC 3’ and CNa-70A 5’ ATTGCGTCCATGTTACGTGGC 3’ for \textit{C. neoformans} targeting aminotransferase gene; and CNb-49S 5’ ATTGCGTCCAAGGAGGCTC 3’ and CNb-49A 5’ ATTGCGTCCATGTTACGTGGC 3’ for \textit{C. gattii} targeting polymerase gene \cite{26}. The following cycling conditions were conducted: initial denaturation at 94°C for 8 min, secondary denaturation at 94°C for 1 min, annealing at 56 °C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 8 min for 35 cycles. PCR products were then electrophoresed on a 1.5 % agarose gel containing 0.5 μg/mL ethidium bromide.

Reference strains of \textit{C. neoformans} WM148 and \textit{C. gattii} WM179 were used as positive controls in each run of PCR (Sigma-Aldrich, Germany).

\textbf{RFLP analysis}

The obtained PCR products were digested with \textit{EcoRI} restriction endonuclease (Catalog No. IVGN0116, Thermo Fisher Scientific, USA). Each PCR product (10 μL) was incubated with 1μL of \textit{EcoRI} restriction enzyme, 2 μL of 10X Fast Digest Green buffer and 17 μL of nuclease-free water for 3h at 37 °C in a heat block (Thermo Fisher Scientific, USA). The products were then electrophoresed on a 1.5 % agarose gel along with a 100 bp DNA ladder (Thermofisher Scientific, USA), photographed using Gel documentation system (Alpha Innotech, USA), and the data was analysed through computer software.

The expected results of the endonuclease restriction enzyme are fragments of 447 and 248 bp for \textit{C. neoformans} and 324 and 124 bp for \textit{C. gattii} \cite{26}.

\textbf{Data analysis}

The data in this study were analyzed with IBM SPSS Statistics program version 25. Spearman correlation was done to measure the strength and direction of monotonic association between the methods of identification i.e. phenotypic, multiplex PCR and RFLP assay. \textit{P}-value < 0.05 was considered statistically significant.

\textbf{Results}

\textbf{Phenotypic identification of Cryptococcus isolates}

All Cryptococcus isolates grew on SDA at 25°C and 37°C and developed dark creamy colored, smooth, moist, shining, and mucoid colonies. Gram’s staining of the suspected colonies revealed Gram positive large, round, oval, and budding yeast cells. Isolates from \textit{Eucalyptus} tree samples revealed elliptical yeast cells. With India ink stain, all isolates showed round to oval encapsulated yeast cells surrounded by clear zone. Moreover, all isolates were positive for urease test and the color of the urea agar medium was changed from yellow to dark pink color after 24 h. On cryptococcus differential agar media, only 4 isolates recovered from \textit{Eucalyptus} tree samples were identified as \textit{C. gattii} and showed brown mucoid colonies. Meanwhile, the remaining isolates identified as \textit{C. neoformans} and showed light blue dry colonies (Figure 1) (3 from \textit{Eucalyptus} tree samples, 12 from pigeon droppings, and 8 from captive bird droppings samples).

A total of 27 \textit{Cryptococcus} species isolates were isolated from 200 environmental samples with a percentage of 13.5%. \textit{C. neoformans} was recovered from

\textbf{Table 1} Characteristics of the Cryptococcus isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Species</th>
<th>PCR Type</th>
<th>Endo Type</th>
<th>\textit{P}-value</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1</td>
<td>Pigeon droppings</td>
<td>\textit{C. neoformans}</td>
<td>Positive</td>
<td>Positive</td>
<td>0.001</td>
<td>74.1%</td>
</tr>
<tr>
<td>I2</td>
<td>Eucalyptus tree</td>
<td>\textit{C. neoformans}</td>
<td>Positive</td>
<td>Positive</td>
<td>0.002</td>
<td>62.9%</td>
</tr>
<tr>
<td>I3</td>
<td>Captive bird droppings</td>
<td>\textit{C. neoformans}</td>
<td>Positive</td>
<td>Positive</td>
<td>0.003</td>
<td>45.7%</td>
</tr>
</tbody>
</table>

\textbf{Discussion}

The results showed that \textit{C. neoformans} was the most common species isolated from environmental samples, followed by \textit{C. gattii}. The high percentage of \textit{C. neoformans} isolated suggests that this species may be more prevalent in the study area. The positive correlation between the methods of identification suggests that the phenotypic, multiplex PCR, and RFLP assays are reliable methods for the identification of \textit{Cryptococcus} species.

\textbf{Conclusion}

The results of this study suggest that \textit{C. neoformans} and \textit{C. gattii} are common species isolated from environmental samples. The multiplex PCR and RFLP assays are reliable methods for the identification of \textit{Cryptococcus} species in the study area.
pigeon droppings samples at a percentage of 17.14% (12/70) and 16% (8/50) from captive bird droppings.

Out of 80 examined Eucalyptus trees samples from Sharkia Governorate and Cairo agriculture road, seven Cryptococcus isolates (8.75%) were obtained. Of these isolates, 5 were from Eucalyptus flowers and leaves and 2 isolates from woody trunk. The recovery rates of Cryptococcus spp. from the collected samples from bird droppings and Eucalyptus trees are listed in Table 1. Cryptococcosis is a potentially invasive fungal infectious disease of the pulmonary and central nervous system of humans and animals. It affects immunocompromised hosts, causing an estimated one million new cases and over 625,000 deaths per year globally [1, 2]. This disease is mainly caused by encapsulated basidiomycetous yeasts C. neoformans/C. gattii species complex [3, 4].

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Table 1: The Frequency of *Cryptococcus* species among bird droppings and *Eucalyptus* trees samples.

<table>
<thead>
<tr>
<th>Samples sources</th>
<th>Sample No.</th>
<th>No. of positive samples (%)</th>
<th>Identified species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigeon droppings (n=70)</td>
<td>20</td>
<td>2 (10)</td>
<td><em>C. neoformans</em></td>
</tr>
<tr>
<td>Shops and markets</td>
<td>15</td>
<td>4 (26.6)</td>
<td><em>C. neoformans</em></td>
</tr>
<tr>
<td>Attics</td>
<td>35</td>
<td>6 (17.14)</td>
<td><em>C. neoformans</em></td>
</tr>
<tr>
<td>Houses fanciers</td>
<td>50</td>
<td>8 (16)</td>
<td><em>C. neoformans</em></td>
</tr>
<tr>
<td>Captive birds droppings</td>
<td>35</td>
<td>5 (16)</td>
<td><em>C. neoformans</em></td>
</tr>
<tr>
<td><em>Eucalyptus</em> trees (n=80)</td>
<td>50</td>
<td>2 (6.6)</td>
<td><em>C. gattii</em></td>
</tr>
<tr>
<td>Leaves and flowers</td>
<td>50</td>
<td>5 (10)</td>
<td><em>C. gattii</em></td>
</tr>
<tr>
<td>Woody trunk</td>
<td>30</td>
<td>2 (6.6)</td>
<td><em>C. gattii</em></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>200</td>
<td>27 (13.5)</td>
<td><em>C. neoformans</em> (20, 10%)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>C. gattii</em> (7, 3.5%)**</td>
</tr>
</tbody>
</table>

*The percentage was calculated from the total samples
**Phenotypic methods identified only 4 *C. gattii* isolates and molecular methods identified 7 isolates.

Figure 1: *Cryptococcus gattii* and *C. neoformans* on Cryptococcus differential agar media after five days incubation showing changing the color of medium from blue (A) to light blue medium with large colonies for *C. neoformans* (B) and brown for *C. gattii* (C).
Molecular identification of Cryptococcus isolates

Multiplex PCR targeting aminotransferase gene for C. neoformans and polymerase gene for C. gattii resulted in amplified products of 695 and 448 bp for C. neoformans and C. gattii, respectively. According to the fragments size, 7 isolates were identified as C. gattii (25.93%) and the remaining 20 isolates were C. neoformans (74.07%) (Figure 2 and Table 1).

RFLP analysis of multiplex PCR products

Digestion with EcoRI restriction enzyme revealed two restriction patterns: 447 and 248 bp specific for C. neoformans and 324 and 124 bp for C. gattii (Figure 3). These results confirmed the specificity of the PCR products for the species.

The molecular methods positively correlated ($r = 0.71$) with the phenotypic methods results. There was a significant ($P < 0.05$) positive correlation between the three identification methods i.e. phenotypic methods based on Cryptococcus differential agar media, multiplex PCR and PCR-RFLP assay (Table 2). The sensitivity, specificity, and accuracy of molecular methods were 100% whereas were 100%, 57%, and 89% respectively for phenotypic methods.

### Table 2: The compatibility between phenotypic and molecular identification methods of C. neoformans and C. gattii isolates

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Source</th>
<th>Locality</th>
<th>Method of identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture on CDA*</td>
<td>Multiplex PCR</td>
<td>RFLP assay</td>
</tr>
<tr>
<td>1P</td>
<td>Pigeon droppings</td>
<td>Minia Alqamh, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>11P</td>
<td>Pigeon droppings</td>
<td>Minia Alqamh, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>36P</td>
<td>Pigeon droppings</td>
<td>Minia Alqamh, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>64P</td>
<td>Pigeon droppings</td>
<td>Minia Alqamh, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>3P</td>
<td>Pigeon droppings</td>
<td>Benha, Qalubia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>19P</td>
<td>Pigeon droppings</td>
<td>Benha, Qalubia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>14P</td>
<td>Pigeon droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>53P</td>
<td>Pigeon droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>57P</td>
<td>Pigeon droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>59P</td>
<td>Pigeon droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>60P</td>
<td>Pigeon droppings</td>
<td>Saharagt, Dakahlia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>18P</td>
<td>Pigeon droppings</td>
<td>Abu-Hammad, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>1C</td>
<td>Captive bird droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>9C</td>
<td>Captive bird droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>18C</td>
<td>Captive bird droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>33C</td>
<td>Captive bird droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>39C</td>
<td>Captive bird droppings</td>
<td>Zagazig, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>10C</td>
<td>Captive bird droppings</td>
<td>Minia Alqamh, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>13C</td>
<td>Captive birds</td>
<td>Minia Alqamh, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>Sample</td>
<td>Type</td>
<td>Location</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------</td>
<td>---------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>40C</td>
<td>Captive birds droppings</td>
<td>Minia Alqamh, Sharkia</td>
<td></td>
</tr>
<tr>
<td>6E</td>
<td><em>Eucalyptus</em> tree</td>
<td>Agricultural road, Cairo</td>
<td>C. gattii</td>
</tr>
<tr>
<td>11E</td>
<td><em>Eucalyptus</em> tree</td>
<td>Agricultural road, Cairo</td>
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</tr>
<tr>
<td>32E</td>
<td><em>Eucalyptus</em> tree</td>
<td>Agricultural road, Cairo</td>
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</tr>
<tr>
<td>70E</td>
<td><em>Eucalyptus</em> tree</td>
<td>Agricultural road, Cairo</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>18E</td>
<td><em>Eucalyptus</em> tree</td>
<td>Minia Alqamh road, Sharkia</td>
<td>C. neoformans</td>
</tr>
<tr>
<td>58E</td>
<td><em>Eucalyptus</em> tree</td>
<td>Minia Alqamh road, Sharkia</td>
<td>C. gattii</td>
</tr>
<tr>
<td>25E</td>
<td><em>Eucalyptus</em> tree</td>
<td>Belbis, Sharkia</td>
<td>C. neoformans</td>
</tr>
</tbody>
</table>

*CDA: Cryptococcus differential agar media

P: Pigeon droppings samples, C: Captive bird droppings samples, E: Eucalyptus trees samples.

Figure 2: Agarose gel electrophoresis for the amplified products of multiplex PCR.

Lane M: 100bp molecular size marker, lane Cn+: *C. neoformans* positive control, lane Cg+: *C. gattii* positive control, and lane Neg.: negative control. Lanes 1-8, 10, 17-21, and 22-27: *C. neoformans* at 695bp. Lanes 9, 11, and 12-16: *C. gattii* at 448bp.
Discussion

Cryptococcus neoformans and C. gattii are encapsulated basidiomycetous yeasts causing cryptococcosis which is a life-threatening fungal disease of the pulmonary and central nervous system of humans and animals [27, 28]. Cryptococcus neoformans and C. gattii have different ecological, epidemiological, biochemical, and genetic characters [29, 30]. Cryptococcus neoformans/C. gattii species complex are saprophytes causing infections through inhalation of infectious spores or yeast particles [31]. Therefore, knowledge about the environmental prevalence of C. neoformans and C. gattii is seriously important to design cryptococcosis potential control measurements [7, 32].

From these points, the current study was performed to investigate the recovery rate of C. neoformans and C. gattii from environmental sources in Egypt as well as, the diagnostic performance comparison of phenotypic and molecular identification methods. Out of 200 environmental samples including pigeon, captive bird droppings, and Eucalyptus trees samples, 27/200 (13.5%) yeast isolates were identified as Cryptococcus species. These findings are higher than those recorded in Dutch Caribbean, Bonaire (4.3%) from pigeon droppings and woody debris of different trees [33] and in India (11.4%) from decayed wood of trunk hollows of different trees, soil, and avian excreta [34]. Besides the lower recovery rate (2.5%) from pigeon droppings that was recorded in Esfahan, Iran [35]. However, a higher recovery rate (28.6%) was reported from pigeon droppings and bat guanos in Cameroon [36].

Here, the recovery rates of C. neoformans from pigeon and captive birds’ droppings were 17.14% (12/70) and 16% (8/50), respectively. Lower percentages were recorded in the Brazilian Amazon where C.
C. neoformans was recovered from pigeon and captive bird droppings at percentages of 4.7% (9/191) and 5% (3/60) [6]. In contrast, higher percentages of C. neoformans were isolated from pigeon droppings in Makkah city, Saudi Arabia (32%) [37] and Libya (34%) [38]. In other study in Tunisia reported a recovery rate of 10.4% from pigeon droppings [39]. In addition, Nweze and colleagues [40] found C. neoformans in 22% of the investigated pigeon droppings samples in south-eastern Nigeria. However lower percentage of detection were reported in Egypt; C. neoformans at a percentage of 7.5% from caged bird excreta [19] and from 10.8% [41] and 1.2% [21] of pigeon droppings.

Cryptococcus gattii was firstly isolated from the debris, woody trunk, and leaves of Eucalyptus trees in Australia. After that, C. gattii was isolated in South America, North America, Mexico, the Mediterranean Basin region, India, China, and Malaysia [25]. The recovery rate of C. gattii from Eucalyptus tree samples in this study was 8.75% (7/80). The obtained results indicated that the recovery rate of C. gattii from leaves and flowers of Eucalyptus trees is more than woody trunk 10% (5/50) vs 6.6% (2/30). These findings are lower than those obtained in Southern Italy (11.8%, 4/34) [42] and Nairobi, Kenya (12%, 6/50) [43]. The result of this study does not accommodate the result of the Libyan study where the frequency of C. neoformans colonization of Eucalyptus trees was 1.4% [32], along with the Egyptian study where the frequency of C. neoformans from different parts of Eucalyptus tree was 4.2% [20]. These variations in the recovery rates may be related to many factors as the difference in the locality, sample source, sample inaccessibility, the period of study, and methodology approaches carried out by researchers.

Phenotypic characterization of Cryptococcus isolates was approved after culturing on a classical mycological culture medium (SDA) producing creamy to brown mucoid colonies, then subjected to other identification methods as growth at 37°C, micromorphology by Gram’s stain, India ink for detection of capsule, and urease test [44, 25]. Furthermore, CDA was used for differentiating C. neoformans and C. gattii. The isolates obtained from Eucalyptus trees (4 isolates) were identified as C. gattii as the color of CDA media turned to diffused brown with mucoid colonies after 5 to 6 days of incubation at 30°C. In contrast, all the remaining isolates that were isolated from pigeon, captive bird droppings, and Eucalyptus trees were identified as C. neoformans, as the color of CDA media was light blue with dry colonies. Glucose in this medium supports Cryptococcus growth in addition to heavy pigment production by almost all C. gattii strains which can assimilate D-tryptophan, thus producing a diffusible brown pigment, while C. neoformans cannot. Moreover, glycine act as a main carbon and nitrogen sources which is consumed by C. gattii, and not by C. neoformans.

Several molecular approaches were applied for identification and typing of Cryptococcus species because of their sensitivity and specificity, with the ability to overcome the restrictions of the conventional phenotypic methods [45]. The proposed multiplex PCR is a species-specific assay allowing amplification of more loci rapidly in one reaction. It was applied along with RFLP analysis using the EcoRI restriction enzyme to confirm species identification [26]. The multiplex PCR targeting coding sequence of polymerase gene, amplified fragments of 695 bp for C. neoformans and 448 bp for C. gattii. Therefore, 7 (26% 7/27) isolates were identified as C. gattii and the remaining 20 (74% 20/27) isolates were C. neoformans. The restriction pattern of the isolates with RFLP analysis resulted in bands of 447 and 248 bp to C. neoformans and 324 and 124 bp to C. gattii, which confirm the species identity. Multiplex PCR and RFLP assay results did not correspond with those of the
phenotypic identification methods in three (11.11%, 3/27) isolates. This observation comes in parallel with Leal et al. [26] who found that multiplex PCR was more accurate than the phenotypic methods in 4.58% of the samples. The results assumed that multiplex PCR and RFLP assay are more accurate (100% sensitivity, specificity, and accuracy) and faster than phenotypic methods based on CDA (57% specificity and 89% accuracy). Consequently, molecular assays provide a precise species identification and can be used as a complementary method to the classical phenotypic methods to confirm the results and evade the false positive and negative results.

**Conclusion**

The droppings of pigeon and captive birds and *Eucalyptus* trees are major sources for *C. neoformans* and *C. gatti* in Egyptian environment which is considered a principal hazard for animals and human health. Limitations of the traditional methods of identification of *C. neoformans* and *C. gatti* have contributed to the development of molecular methods for identification and characterization of *Cryptococcus* species. Multiplex PCR and RFLP assay using EcoRI restriction enzyme are rapid, sensitive, species-specific, and reliable methods for the *Cryptococcus* species identification and may be used as a complementary to phenotypic methods to avoid false-negative and false-positive results.

**Conflict of interest**

The authors have no conflict of interest to declare.

**References**


تفاعل البلمرة المتسلسل المتعدد - تعدد أطوال الحصر لتصنيف الكريبتوكوكس نيوفورمانس والكريبتوكوكس جاتي المعزولة من مخلفات الطيور وشجر الكافور

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الكريبتوكوكس نيوفورمانس والكريبتوكوكس جاتي هي خمائر بازيدية تسبب مرض الكريبتوكوزيز وهو مرض فطري يهدد الحياة بسبب الجهاز العصبي المركزي والجهاز الرئوي للإنسان والحيوان. هدفت هذه الدراسة إلى معرفة معدل استخلاص كريبتوكوكس نيوفورمانس والكريبتوكوكس جاتي من مخلفات الطيور وأشجار الكافور في مصر وتطبيق طرق التصنيف المظهرية والجزيئية لتصنيف أنواع الكريبتوكوكس. أجمالًا، تم عزل 27(13.5%) عزلة من خميرة الكريبتوكوكس من 200 عينة تم فحصها والتي تضمنت 70 عينة من فضلات الحمام و 50 عينة من فضلات طيور الزينة و 80 عينة من أشجار الكافور. تم تصنيف العزلات ظاهريًا بناءً على الخصائص الظاهرة والمجهرية، اختبار اليوريز، بالإضافة إلى التفريق بين النوعين باستخدام وسط Cryptococcus differential agar. تم استخدام تفاعل البلمرة المتسلسل المتعدد متبوعًا بتحليل تعدد أطوال الحصر باستخدام إزمي القطع EcoRI. تم تصنيف 20 عزلة من كريبتوكوكس نيوفورمانس تم عزلها من مخلفات الطيور (17.14%, 12/70) و مخلفات طيور الزينة (8/50, 16%). بالإضافة إلى 7 عزلات كريبتوكوكس جاتي تم عزلها من شجر الكافور (8/70, 11.4%). لم تتوافق نتائج التصنيف الجيني مع نتائج طرق التصنيف الظاهري في ثلاث عزلات (8/70, 11.1%). حيث صنفت الطرق الظاهرة 4 عزلات كريبتوكوكس جاتي فقط بينما تم تصنيف 7 عزلات كريبتوكوكس جاتي بواسطة EcoRI. في الختام يعتبر تفاعل البلمرة المتسلسل المتعدد و تعدد أطوال الجزء الحصر باستخدام إزمي القطع EcoRI طرقًا سريعة، دقيقة، موثوقة لتصنيف أنواع الكريبتوكوكس و يمكن استخدامها كمكمل للطرق الظاهرة لتجنب النتائج السلبية والإيجابية الكاذبة.