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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 *Eco*RI 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.

Keywords: *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℃ [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 25ºC and 37ºC 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 ºC, and biochemical test for urease activity [24, 25]. Finally, cryptococcus differential agar media (Himedia, India) was used for differentiation of *C. neoformans* and *C. gattii* on the basis of assimilation of tryptophan and glycine as carbon and nitrogen sources.

Molecular identification

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

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′ ATTGCGTCCACCAAGGAGCTC 3′ and CNa- 70A 5′ ATTGCGTCCATGTTACGT GGC 3′ for *C. neoformans* targeting aminotransferase gene; and CNb-49S 5′ ATTGCGTCCAAGGTGTTGTTG 3′ and CNb-49A 5′ ATTGCGTCCATCCAACCG TTATC 3′ for *C. gattii* targeting polymerase gene [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 1.5% agarose gel containing 0.5 µg/mL ethidium bromide.

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

RFLP analysis

The obtained PCR products were digested with *Eco*RI restriction endonuclease (Catalog No. IVGN0116, Thermo Fisher Scientific, USA). Each PCR product (10 µL) was incubated with 1µL of *Eco*RI restriction enzyme, 2 µL of 10X Fast Digest Green buffer and 17 µL of nuclease-free water for 3h at 37 ºC 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 *C. neoformans* and 324 and 124 bp for *C. gattii* [26].

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. *P*-value < 0.05 was considered statistically significant.

Results

Phenotypic identification of Cryptococcus isolates

All Cryptococcus isolates grew on SDA at 25℃ and 37℃ 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 *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 *Eucalyptus* tree samples were identified as *C. gattii* and showed brown mucoid colonies. Meanwhile, the remaining isolates identified as *C. neoformans* and showed light blue dry colonies (Figure 1) (3 from *Eucalyptus* tree samples, 12 from pigeon droppings, and 8 from captive bird droppings samples).

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

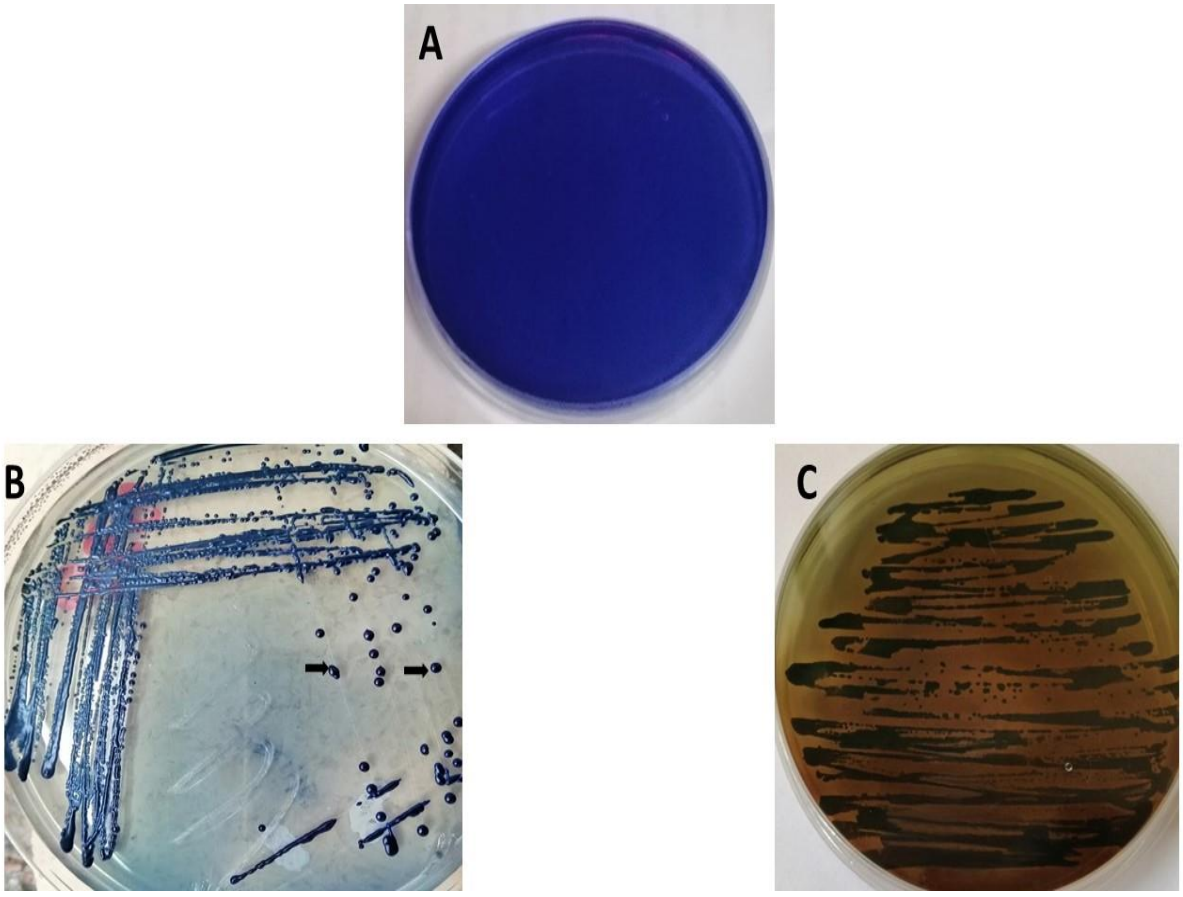

Figure 1: *Cryptococcus gattii* and *C. neoformans* on Cryptococcus differential agar media after five days incubation showing changing the color of medium from blue (A) to light blue medium with large colonies for *C. neoformans* (B) and brown for *C. gattii* (C).

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

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

*The percentage was calculated from the total samples

**Phenotypic methods identified only 4 *C. gattii* isolates and molecular methods identified 7 isolates.

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 *Eco*R I 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

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

*CDA: Cryptococcus differential agar media

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

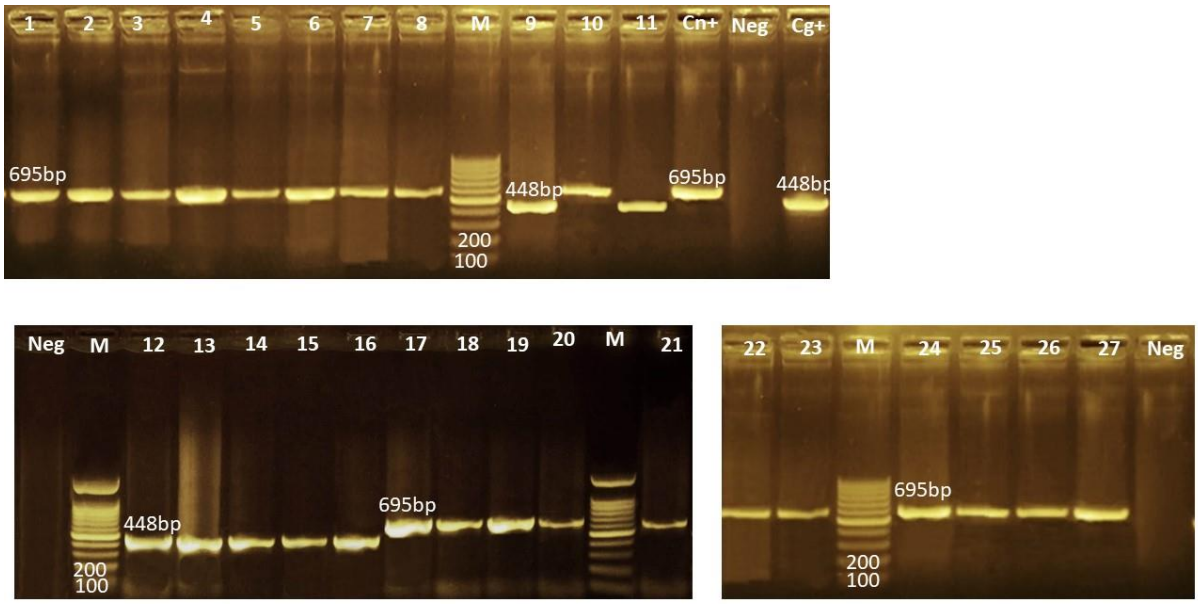

Figure 2: Agarose gel electrophoresis for the amplified products of multiplex PCR.

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.

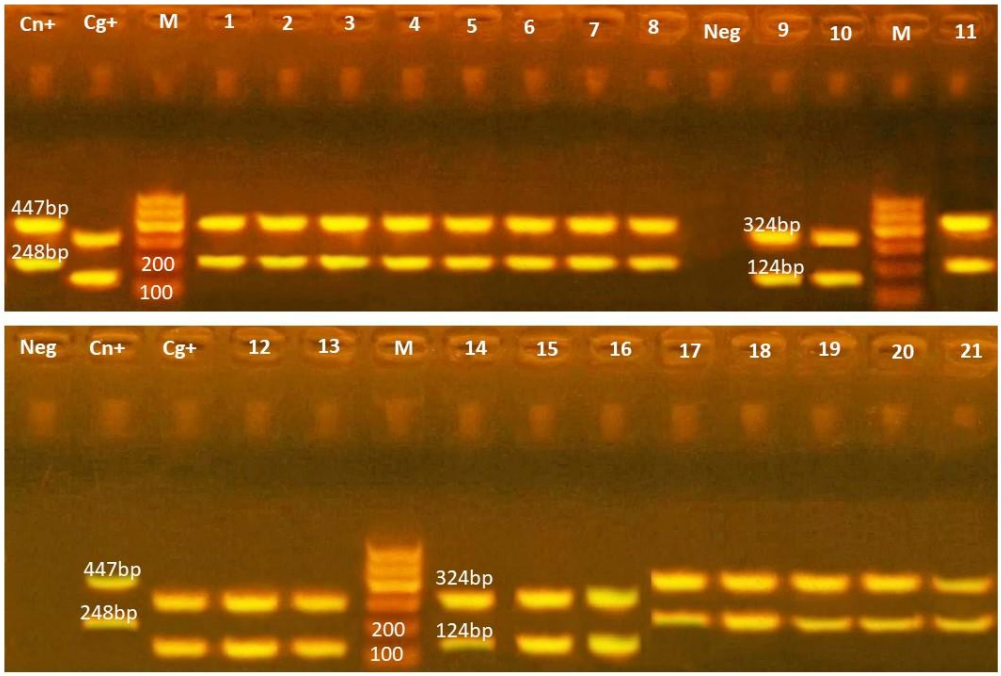

Figure 3: Agarose gel electrophoresis for the amplified products digested with the restriction endonuclease *Eco*RI.

Lane M: 100 bp molecular size marker, Lane Neg.: negative control.
Lane Cg+: control positive for *C. gattii*, and lane Cn+: Control positive for *C. neoformans*.
Lanes 9, 10, and 12-16: restriction pattern of *C. gattii* (124 and 324bp).
Lanes 1-8, 11, and 17-21: restriction pattern of *C. neoformans* (284 and 447bp).

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. 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℃, 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℃. 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 *Eco*R I restriction enzyme to confirm species identification [26]. The multiplex PCR targeting coding sequence of polymerase gene, amplified fragments of 695 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. gattii* have contributed to the development of molecular methods for identification and characterization of *Cryptococcus* species. Multiplex PCR and RFLP assay using *Eco*R I 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

1. Park, B.J.; Wannemuehler, K.A.; Marston, B.J., Govender, N.; Pappas, P.G. and Chiller, T.M. (2009): Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS 23: 525–530.
2. Kurtzman, C.P.; Fell, J.W. and Boekhout, T. (2011): The Yeasts: A Taxonomic Study. 5th Edition, ACM Press, NY.
3. Rajasingham R.; Smith R.M.; Park B.J.; Jarvis J.N.; Govender N.P.; Chiller T.M.;

Denning D.W.; Loyse A. and Boulware DR. (2017): Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. Lancet Infect Dis, 17:873–881.

4. Montoya, M.C.; Magwene, P.M. and Perfect, J.R. (2021): Associations between Cryptococcus Genotypes, Phenotypes, and Clinical Parameters of Human Disease: A Review. J Fungi (Basel), 7:260.
5. Refai, M.; Elhariri, M. and Alarousy, R. (2017): Cryptococcosis in Animals and Birds: A Review. 4: 202-223.
6. Alves, G.S.; Freire, A.K.; Bentes Ados, S.; Pinheiro, JF.; de Souza, JV.; Wanke, B. et al., (2016): Molecular typing of environmental *Cryptococcus neoformans/ C. gattii* species complex isolates from Manaus, Amazonas, Brazil. Mycoses, 59: 509-15.
7. Chowdhary A.; Randhawa H.S.; Prakash A. and Meis J.F. (2011): Environmental prevalence of *Cryptococcus neoformans* and *Cryptococcus gattii* in India: An update. Crit Rev Microbiol, 38: 1–16.
8. Cogliati M (2013): Global molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii*: an atlas of the molecular types. Scientifica, 2013: 675213.
9. Chen S.C.; Meyer W. and Sorrell TC (2014): *Cryptococcus gattii* infections. Clin Microbiol Rev, 27: 980–1024.
10. Silva, D. and Maranhão, F. (2015): Current Status of the Diagnostic and Genomics of *Cryptococcus neoformans/C. gattii* Species Complex. Fungal Genom Biol, 5: 2-5.
11. Klein, K.; Hall, L.; Deml, S.; Rysavy, J.; Wohlfiel, Sh. and Wengenack, N. (2009): Identification of *Cryptococcus gattii* by Use of L-Canavanine Glycine Bromothymol Blue Medium and DNA Sequencing. Journal of clinical microbiology, 47: 3669-72.
12. Meyer W.; Gilgado F.; Ngamskulrungroj P.; Trilles LH.; Hagen F.; Castaneda E. and Boekhout T. (2011): Molecular typing of the *Cryptococcus neoformans/Cryptococcus gattii* species complex. In *Cryptococcus*: From human pathogen to model yeast (ed. Heitman J, et al.), pp. 327–357. ASM, Washington, DC.
13. Sidrim, J.; Costa, A.; Cordeiro, R.; Brilhante, R.S.; Moura, F.E.; Castelo-Branco, D.S.; Neto M.P. and Rocha, MF. (2010): Molecular methods for the diagnosis and characterization of *Cryptococcus*: a review. Can J Microbiol, 56: 445-458.
14. Meyer, W.; Aanensen, D.M.; Boekhout,T.; Cogliati, M.; Diaz, M.R.; Esposto, M.C et al., (2009): Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Med Mycol, 47: 561-70.
15. Feng, X.; Yao, Z. and Liao, W. (2013): Approaches for molecular identification and typing of the *Cryptococcus* species complex: an update. Reviews in Medical Microbiology, 24: 1–6.
16. Bovers, M.; Diaz, M.R.; Hagen, F.; Spanjaard, L.; Duim, B.; Visser, C.E. et al., (2007): Identification of genotypically diverse *Cryptococcus neoformans* and *Cryptococcus gattii* isolates by Luminex xMAP technology. J Clin Microbiol, 45:1874–1883.
17. Trilles, L.; Wang, B.; Firacative, C.; Lazéra Mdos, S.; Wanke, B. and Meyer, W. (2014): Identification of the major molecular types of *Cryptococcus neoformans* and *C. gattii* by hyper branched rolling circle amplification. PLoS ONE, 9: e94648.
18. Hagen, F.; Khayhan, K.; Theelen, B.; Kolecka, A.; Polacheck, I.; Sionov, E. et al., (2015): Recognition of seven species in the *Cryptococcus gattii/Cryptococcus neoformans* species complex. Fungal Genet Biol, 78: 16–48.

19. Elhariri, M.; Hamza, D.; Elhelw, R. and Refai, M. (2015): Lovebirds and cockatiels risk reservoir of *Cryptococcus neoformans*, a potential hazard to human health. J Vet Sci & Med Diagn, 4: 4, 2.

20. Elhariri, M.; Hamza, D.; Elhelw, R. and Refai, M. (2016): Eucalyptus tree: a potential source of *Cryptococcus neoformans* in Egyptian environment. Int J Microbiol, 2016: 1–5.

21. Elkady, E. and Torky, H. 2016: *Cryptococcus neoformans* isolated from pigeon, chicken dropping samples and dust collected from their house on urease hydrolysis. Alex J Vet Sci, 51: 122-128.

22. Li, L.; Wang, J.c. and Zhang, Qq. (2000): Isolation and identification of *Cryptococcus neoformans* from pigeon dropping. J Clin Dermatol, 29: 4: 198-200.

23. Kidd, S.E.; Chow, Y.; Mak, S.; Bach, P.J.; Chen, H.; Hingston, A.O. et al., (2007): Characterization of environmental sources of the human and animal pathogen *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest of the United States. App Environ Microbiol, 73:1433-43.

24. Granados, D.P. and Castañeda, E. (2005): Isolation and characterization of *Cryptococcus neoformans* varieties recovered from natural sources in Bogotá, Colombia, and study of ecological conditions in the area. Microb Ecol, 49: 282-290.

25. Singh, K.; Ilkit, M.; Shokohi, T.; Tolooe, A.; Malik, R. and Seyedmousavi, S. (2018): Cryptococcosis: Emergence of *Cryptococcus gattii* in animals and zoonotic potential. In Seyedmojtaba, S; Sybren, de Hoog; Jacques, G; Paul, E. editors. Emerging and Epizootic Fungal Infections in Animals, Springer, Cham, 249–87.

26. Leal, A.L.; Faganello, J.; Bassanesi, M.C. and Vainstein, M.H. (2008): *Cryptococcus* species identification by multiplex PCR. Med Mycol, 46: 377–383.

27. Perfect, J.R. and Bicanic, T. (2015): Cryptococcosis diagnosis and treatment: what do we know now. Fungal Genet Biol, 78: 49–54.

28. Cogliati, M.; D'Amicis, R.; Zani, A.; Montagna, M. T.; Caggiano, G.; De Giglio, O., et al., (2016): Environmental distribution of *Cryptococcus neoformans* and *C. gattii* around the Mediterranean basin. FEMS yeast research, 16: fow045.

29. Chun, C.D. and Madhani, H.D. (2010): Applying genetics and molecular biology to the study of the human pathogen *Cryptococcus neoformans*. Methods Enzymol, 470: 797–831.3.

30. May, R.; Stone, N.; Wiesner, D.; Bicanic, T. and Nielsen, K. (2016): *Cryptococcus*: from environmental saprophyte to global pathogen. Nat Rev Microbiol, 14: 106-117.

31. Harris, J.; Lockhart, S.; Chiller, T. (2012): *Cryptococcus gattii*: where do we go from here? Med Mycol, 50:113–129.

32. Taha, M.; Tartor, Y.H.; Ibrahim, S.Z. and Abd El-Aziz, R.M. (2020): Molecular typing and susceptibility profile of *Cryptococcus neoformans* and *Cryptococcus gattii* species complex: An updated review. J Anim Health Prod, 9:17-26.

33. Gugnani, H. C.; Hagen, F.; Meis, J. F. and Chakrabarti, A. (2020): Occurrence of *Cryptococcus neoformans* and other yeast-like fungi in environmental sources in Bonaire (Dutch Caribbean). Germs, 10: 195–200.

34. Prakash, A.; Randhawa, H.S.; Khan, Z.U.; Ahmad, S.; Hagen, F.; Meis, J.F. et al., (2018): Environmental distribution of *Cryptococcus* species and

some other yeast-like fungi in India. Mycoses, 61: 305-313.

35. Soltani, M.; Bayat, M.; Hashemi, J.; Zia, M. and Pestehchian, N. (2013): Isolation of *Cryptococcus neoformans* and other opportunistic fungi from pigeon droppings. J Res Med Sci , 18: 56-60.
36. Dongmo, W.; Kechia, F.; Tchuenguem, R.; Nangwat, C.; Yves, I.; Kuiate, J. R. et al., (2016): In vitro antifungal susceptibility of environmental isolates of *Cryptococcus* spp. from the West Region of Cameroon. Ethiop J Health Sci, 26: 555–560.
37. Abulreesh, H.; Organji, S.; Elbanna, Kh.; Osman, G.; Almalki, M. and Abdel-Maledk, A. (2015): First report of environmental isolation of *Cryptococcus neoformans* and other fungi from pigeon droppings in Makkah, Saudi Arabia and in vitro susceptibility testing. Asian Pacific Journal of Tropical Disease, 5: 622-626.
38. Ellabib, M.S.; Aboshkiwa, M.A.; Husien, W.M.; D'Amicis, R. and Cogliati, M. (2016): Isolation, identification and molecular typing of *Cryptococcus neoformans* from pigeon droppings and other environmental sources in Tripoli, Libya. Mycopathologia, 181: 603–8.
39. Mseddi, F.; Sellami, A.; Jarboui, M.A.; Sellami, H.; Makni, F. and Ayadi, A. (2011): First environmental isolations of *Cryptococcus neoformans* and *Cryptococcus gattii* in Tunisia and review of published studies on environmental isolations in Africa. Mycopathologia, 171: 355-60.
40. Nweze, E. I.; Kechia, F. A.; Dibua, U. E.; Eze, C. and Onoja, U. S. (2015): Isolation of *Cryptococcus neoformans* from environmental samples collected in southeastern Nigeria. Rev Inst Med Trop São Paulo, 57: 295-298.
41. Abdel-Razik, M. (2007): Domestic Birds as Carriers of *Cryptococcus neoformans*, the main cause of the life threatening human systemic cryptococcosis. Catrina, 2: 91.-96.
42. Romeo, O.; Scordino, F. and Criseo, G. (2011): Environmental isolation of *Cryptococcus gattii* serotype B, VGI/MATα strains in southern Italy. Mycopathologia, 171: 423-30.
43. Kangogo, M.; Boga, H.; Wanyoike, W. and Bii, C. (2014): Isolation and characterisation of *Cryptococcus neoformans* and *Cryptococcus gattii* from environmental sources in Nairobi, Kenya. East Afr Med J, 91: 281-5.
44. Doering, T.L. (2009): How sweet it is! Cell wall biogenesis and polysaccharide capsule formation in *Cryptococcus neoformans*. Annu Rev Microbiol, 63: 223–247.
45. Dou, H.; Wang, H.; Xie, S.; Chen, X.; Xu, Z. and Xu, Y. (2017): Molecular characterization of *Cryptococcus neoformans* isolated from the environment in Beijing, China. Med Mycol, 55: 737-747.

الملخص العربي

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

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الكريبتوكوكس نيوفورمانس والكريبتوكوكس جاتي هي خمائر بازيدية تسبب مرض الكريبتوكوكوزيز وهو مرض فطري يهدد الحياة يصيب الجهاز العصبي المركزي والجهاز الرئوي للإنسان والحيوان. هدفت هذه الدراسة إلى معرفة معدل استخلاص كريبتوكوكس نيوفورمانس و كريبتوكوكس جاتي من مخلفات الطيور وأشجار الكافور في مصر و تطبيق طرق التصنيف المظهرية والجزيئية لتصنيف أنواع الكريبتوكوكس. إجمالاً، 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 ظاهريا بناء على الخصائص الظاهرية والمجهرية، اختبار اليوريز، بالاضافة الى التفريق بين النوعين باستخدام وسط Cryptococcus differential agar. تم استخدام تفاعل البلمرة المتسلسل المتعدد متبوعا بتحليل تعدد أطوال الحصر باستخدام إنزيم القطع *Eco*RI والذي أكد دقة تفاعل البلمرة المتسلسل المتعدد في تحديد الأنواع لتحديد النوعين. بالتصنيف الجزيئي تم تصنيف 20 عزلة الى كريبتوكوكس نيوفورمانس تم عزلها من مخلفات الطيور (12/70, 17.14%) و مخلفات طيور الزينة (50/8 , 16%)، بالاضافة الى 7 عزلات كريبتوكوكس جاتي تم عزلها من شجر الكافور (80/7 ,8.75%). لم تتوافق نتائج التصنيف الجزيئي مع نتائج طرق التصنيف الظاهري في ثلاث عزلات (11.11٪). حيث صنفت الطرق الظاهرية 4 عزلات كريبتوكوكس جاتي فقط، بينما تم تصنيف 7 عزلات كريبتوكوس جاتي بواسطة الطرق الجزيئية. فى الختام يعتبرتفاعل البلمرة المتسلسل المتعدد و تعدد أطوال جزء الحصر باستخدام إنزيم قطع *Eco*RI طرقا سريعة، حساسة، دقيقة، موثوقة لتصنيف انواع الكريبتوكوكس ويمكن استخدامها كمكمل للطرق الظاهرية لتجنب النتائج السلبية والإيجابية الكاذبة .