Isolation and molecular characterization of Cryptococcus species isolated from pigeon nests and Eucalyptus trees

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Background and Purpose: Cryptococcus species are pathogenic and non-pathogenic basidiomycete yeasts that are found widely in the environment. Based on phenotypic methods, this genus has many species; however, its taxonomy is presently being re-evaluated by modern techniques. The Cryptococcus species complex includes two sibling taxa of Cryptococcus neoformans and Cryptococcus gattii. We aimed to investigate the possible distribution of Cryptococcus species in pigeon nests and Eucalyptus trees in Ilam, Iran, using molecular techniques.

Materials and Methods: Two hundred and seventy-four specimens were collected from pigeon nests and Eucalyptus trees during 2016-2017. All the specimens were sub-cultured on Sabouraud Glucose Agar with chloramphenicol and bird seed agar. For molecular identification, the ITS1-5.8S-ITS2 rDNA region was amplified using the first and fourth internal transcribed spacer (ITS1 and ITS4, respectively) primers. The purified products were applied for cycle sequencing reactions in forward direction with ITS1 primer. The obtained results were analyzed with Chromas 2.3.

Results: Thirty-three out of 186 cultures (17.7%) and 11 out of 88 cultures (12.5%) were positive among pigeon nest and Eucalyptus tree specimens, respectively. Cryptococcus albidus (17.2%), C. albidas var. kuetzingii (3.4%), C. adelensis (3.4%), C. uzbekistanensis (3.4%), and C. neoforans var. grubii (3.4%) were isolated from pigeon nests, and Cryptococcus adelensis (25%) was the only Cryptococcus species isolated from Eucalyptus trees.

Conclusion: The presence of pigeons and Eucalyptus trees in the vicinity of some particular places such as rest homes and hospitals should be considered as a risk factor for the immunocompromised population.

Keywords: Cryptococcus gattii, Cryptococcus neoformans, Eucalyptus trees, ITS sequencing, Pigeon nests

Introduction

Cryptococcus species are environmental basidiomycete yeasts that are found widely in the environment. The majority of the species live in the soil and are not pathogenic. According to the previous studies, the Cryptococcus genus includes more than 35 species [1, 2]; however, after more than 15 years of phenotypic and molecular studies, a proposal for a taxonomic revision was made. The genus Cryptococcus in its current concept encompasses the dimorphic yeasts C. biallisporus, C. decagattii, C. amyloleptus, C. deneofomans, C. deuteroagattii, C. gattii, C. neoformans, and C. tetragattii, as well as the filamentous species C. luteus and C. depauperatus [3]. Phylogenetic analysis revealed that Cryptococcus species complex includes two sibling taxa of Cryptococcus gattii and Cryptococcus neoformans. Both species are major of human and animal pathogens. Cryptococcus neoformans was first isolated by Sanfelice in 1894 in Italy from peach juice. The environmental origin of C. neoformans was obscure until Emmons reported pigeon nests and droppings as the main source of this fungal pathogen [4, 5]. Pigeons can even transmit C. neoformans on their feathers, beaks, and legs. The fungus is not the common flora of soil, and it is usually isolated from regions that are in contact with pigeons, turkeys, chickens, and other avian species [6, 7].

Cryptococcus gattii, formerly known as Cryptococcus neoformans var. gattii, is another species of Cryptococcus found principally in the tropical and subtropical areas. It is a geographically limited fungus that has been found frequently in soil debris, especially regions with certain trees like oaks and Eucalyptus [8]. Northern Australia and Papua New Guinea have the
highest incidence of Cryptococcus gattii infection, but many cases of infection have also been reported from other areas including India, Vancouver Island in Canada, Brazil, and Washington State in the United States.

Unlike Cryptococcus neoformans, that is remarkably associated with human immunodeficiency virus (HIV) infection or other immunodeficiency disorders, Cryptococcus gattii can cause infection in healthy individuals, as well [9]. Soltani et al. [10] reported 3 out of 120 samples (2.5%) as the frequency of Cryptococcus neoformans in towers of urban areas of Isfahan, Iran, in a period of nine months by using RapID Yeast Plus System and canavanine glycine bromothymol blue medium (CGB) test. Hedayati et al. [11] evaluated the isolation of Cryptococcus neoformans from swallow (Hirundo rustica) excreta in two northern cities of Iran. They isolated Cryptococcus neoformans from 5/97 (5.2%) of collected samples. Khoravi [12] sought for Cryptococcus neoformans among 983 samples of pigeon droppings from various regions in northern Iran including Rasht, Ramsar, Babol, Sari, and Gorgan. They used phenotypic methods, namely culture on Guizotia abyssinica creatinine agar and CGB agar, and Cryptococcus neoformans was isolated from 17.8% of the specimens. Badali et al. [13] isolated Cryptococcus neoformans genotype AFLP1/VNI from a 49-year-old HIV-positive female in Sari, Iran, by sequencing the internal transcribed spacer (ITS) rDNA region. Salehei et al. [14] collected 156 samples of flowers, fruits, leaves, and barks of Eucalyptus trees and soil underneath Eucalyptus trees over a period of six months from various parts of Ahvaz, Iran. They used the traditional tests such as sub-culturing on Sabouraud Dextrose Agar, urease production, and growth at 37°C in the presence of capsule around yeasts using Indian ink preparation for the identification of Cryptococcus gattii, but they could not isolate Cryptococcus gattii from Eucalyptus trees and soil in Ahvaz. Due to the limited data on these potential pathogens in Ilam (a western province of Iran), the present study aimed to investigate the feasible distribution of Cryptococcus neoformans and Cryptococcus gattii in pigeon nests and Eucalyptus trees, respectively.

Materials and Methods

Sampling: From November 2016 to March 2017, 274 specimens were obtained as follows:

A) Pigeon nests: One hundred and eighty-six samples were taken from pigeon nests in pet shops, houses of pigeon fanciers, and attics. Then, 15 g of pigeon droppings was collected by a sterile spade and put in zip lock bags. Laboratory processing of the samples was performed on the same day. The specimens were processed in accordance with Shields and Ajello method [7, 15]. Briefly, a suspension of each pigeon droppping was made in sterile saline solution 1:10 (w/v), and chloramphenicol (0.3 mg/ml) or streptomycin (2 mg/ml) (Sigma-Aldrich, Germany) was added to the suspension and mixed for 10 min and allowed to settle down for 40 min. Ten microliters of the supernatant of each suspension was added to Petri dishes containing Guizotia abyssinica agar (bird seed agar) (BIOMARK, India) and incubated at 30-32°C for three weeks. Coffee-colored colonies were considered Cryptococcus neoformans. Each brown colony was examined microscopically using 10% KOH with methylene blue and Gram stain. Definitive identification of isolates was performed using DNA sequencing technique.

B) Eucalyptus trees: Eighty-eight samples containing soil (30 g), leaves (20 g), woody debris (20 g), and tree bark (10 g) were put in sterile transport bags and stored for the following steps of the experiment. Four grams of each specimen was suspended in sterile water, vortexed, and allowed to settle down for about 10 min. Afterwards, 200 µl of each sample was transferred to Sabouraud Glucose Agar with chloramphenicol (Sigma-Aldrich, Germany), and all the yeast colonies were applied for sequencing [16, 17].

Molecular identification

DNA extraction: This process was conducted using boiling method [18, 19]. Briefly, 2-3 fresh colonies were suspended in 40 µl of double distilled water, boiled gently for 10-15 min, and then centrifuged for 7 min at 6000 rpm. The supernatant was used for polymerase chain reaction (PCR).

PCR: The ITS1-5.8S-ITS2 rDNA region was amplified using ITS1 (5’-TCC GTA GGT GAA CCT GCG G-3’) and ITS4 (5’-TCC TCC GCT TAT GGA TAT GC-3’) primers [20]. The PCR conditions were as follows: denaturation of DNA at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, with a final extension phase at 72°C for 7 min. The amplicons were purified by QIAquick PCR Purification Kit (Qiagen, USA). Seven microliters of each PCR product was run onto 1.5% agarose gel and electrophoresed in Tris–borate–EDTA buffer (90 mM boric acid, 2 mM 127 ethylenediaminetetraacetic acid, and 90 mM Tris) at 10 V/cm for 120 min, and 0.5 µg/ml of ethidium bromide (Sigma-Aldrich, Germany) was used for staining.

Sequencing: The purified amplicons were applied for cycle sequencing reactions in forward direction (Bioneer, Korea) with ITS1 primer. The sequence results were analyzed with Chromas 2.3 (http://chromas.software.informer.com/2.3/).

Results

Thirty-three out of 186 cultures (17.7%) and 11 out of 88 cultures (12.5%) were positive among pigeon nest and Eucalyptus tree specimens, respectively. Three positive samples of Eucalyptus trees and four positive cultures of pigeon nests had weak bands on agarose gel and were excluded from the investigation. Eighteen positive pigeon nest specimens (62.1%) were obtained from pet shops, 6 specimens (20.6%) from attics, and 5 specimens (17.2%) from houses of pigeon fanciers. Positive cultures of Eucalyptus trees belonged to leaves (no. 5: 62.5%), dust and debris around the
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trees (no. 2; 25%), and barks (no. 1; 12.5%). Cryptococcus albidus (Naganishia albida; 17.2%), C. albidus var. kuetzingii (Naganishia uzbekistanensis; 3.4%), C. adeliensis (N. adeliensis; 3.4%), C. uzbekistanensis (3.4%), and C. neoformans var. grubii (genotype AFLP1/VNI; 3.4%) were isolated from pigeon nests. Cryptococcus adeliensis (25%) was the only Cryptococcus species isolated from Eucalyptus trees. Figure 1 shows the frequency of Cryptococcus species in the present study. Table 1 presents all the yeasts isolated from both pigeon nests and Eucalyptus trees with their accession numbers.

Figure 1. The frequencies of Cryptococcus species in the present study

Table 1. Yeast isolates obtained from pigeon nests and Eucalyptus trees and identified using polymerase chain reaction sequencing (rDNA ITS region)

| No. | Location                | Isolate                                                        | Accession number |
|-----|-------------------------|----------------------------------------------------------------|------------------|
| 1   | Pet shops               | C. albidus var. kuetzingii (Naganishia uzbekistanensis)       | MG020688         |
| 2   | Pet shops               | C. adeliensis (N. adeliensis)                                  | MG020689         |
| 3   | Pet shops               | Hanseniaspora avarum                                          | MG020690         |
| 4   | Pet shops               | Cryptococcus uzbekistanensis                                   | MG020691         |
| 5   | Pet shops               | C. albidus (N. albida)                                         | MG020692         |
| 6   | Pet shops               | C. albidus (N. albida)                                         | MG020693         |
| 7   | Pet shops               | Filobasidium uniguttulatum                                    | MG020694         |
| 8   | Pet shops               | Debaryomyces shansenii                                        | MG020695         |
| 9   | Pet shops               | Debaryomyces hansenii                                         | MG020696         |
| 10  | Pet shops               | C. albidus (N. albida)                                         | MG020697         |
| 11  | Pet shops               | C. albidus (N. albida)                                         | MG020710         |
| 12  | Pet shops               | C. albidus (N. albida)                                         | MG020711         |
| 13  | Pet shops               | Debaryomyces hansenii                                         | MG020712         |
| 14  | Pet shops               | Rhodotorula mucilaginosa                                       | MG020720         |
| 15  | Pet shops               | Candida albicans                                               | MG020713         |
| 16  | Pet shops               | Meyerozyma guilliermondii                                     | MG020719         |
| 17  | Pet shops               | Meyerozyma guilliermondii                                     | MG020717         |
| 18  | Pet shops               | Candida albicans                                               | MG020714         |
| 19  | Attic                   | C. neoformans var. grubii (genotype AFLP1/VNI)                | MG020698         |
| 20  | Attic                   | Debaryomyces hansenii                                         | MG020699         |
| 21  | Attic                   | Debaryomyces hansenii                                         | MG020700         |
| 22  | Attic                   | Debaryomyces hansenii                                         | MG020701         |
| 23  | Attic                   | Debaryomyces hansenii                                         | MG020702         |
| 24  | Attic                   | Candida albicans                                               | MG020703         |
| 25  | Houses of pigeon fanciers| Debaryomyces hansenii                                         | MG020704         |
| 26  | Houses of pigeon fanciers| Debaryomyces hansenii                                         | MG020705         |
| 27  | Houses of pigeon fanciers| Candida albicans                                               | MG020706         |
| 28  | Houses of pigeon fanciers| Meyerozyma guilliermondii                                     | MG020707         |
| 29  | Houses of pigeon fanciers| Candida albicans                                               | MG020715         |

| No. | Location                | Isolate                                                        | Accession number |
|-----|-------------------------|----------------------------------------------------------------|------------------|
| 1   | Leaves                  | Trichosporon asahii                                            | MG020686         |
| 2   | Leaves                  | Rhodotorula mucilaginosa                                       | MG020687         |
| 3   | Leaves                  | C. adeliensis (N. adeliensis)                                  | MG020708         |
| 4   | Leaves                  | C. adeliensis (N. adeliensis)                                  | MG020709         |
| 5   | Leaves                  | Candida albicans                                               | MG020718         |
| 6   | Bark                    | Candida albicans                                               | MG020721         |
| 7   | Dust and debris around the trees | Candida albicans                                           | MG020722         |
| 8   | Dust and debris around the trees | Meyerozyma guilliermondii                                     | MG020716         |
Discussion

Cryptococcus neoformans is a common yeast-like fungus in bird droppings, including pigeon, and soil contaminated with bird excrements. Although pigeon nests are considered as the natural habitat of C. neoformans, this species has been isolated from other sources like tree trunk hollows, bark, and decaying materials, as well [21]. In the present study, we isolated Cryptococcus adeliensis from Eucalyptus trees, but C. neoformans was not obtained from various parts of Eucalyptus trees. C. adeliensis was formerly described as a novel Cryptococcus species obtained from algae in Antarctica; this species is incapable of fermentation as is representative of the Cryptococcus species [22]. In the present investigation, we obtained three C. adeliensis isolates from pigeon droppings in pet shops (1 isolate) and the leaves of Eucalyptus trees (2 isolates).

Rimek et al. [23] reported the first case of meningitis caused by Cryptococcus adeliensis in a German patient with acute myeloid leukemia. They completed phenotypic tests and identified isolates by sequencing the D1/D2, ITS 1, and ITS 2 regions of the 26S rDNA. In 2005, Tintelnot and Losert [24] isolated C. adeliensis from both clinical and environmental specimens. They isolated C. adeliensis from pigeon droppings collected from an urban recreation park (Tegel Lake in Berlin) and from a pigeon breeding facility near Hanover, Germany. They also isolated C. adeliensis from a lung biopsy of an adult male and from the oral cavity of an 8-year-old girl with HIV infection. Cryptococcus adeliensis grows in smooth and cream-colored colonies and is misidentified as C. albidos due to the high variability of its phenotypic characteristics. Therefore, molecular techniques such as sequence analysis for C. albidos clade are essential to identify C. adeliensis, as was performed in the present study.

In 2016, Borhani and Rahimian [25] isolated C. adeliensis from cankers on stone fruits in Khorasan provinces, Iran. They showed stem canker caused by C. adeliensis is a newly emerged disease of fruit trees characteristicly comparable to the bacterial canker disease [26]. Cryptococcus albidos was another species in the Cryptococcus genus isolated in this investigation. It is an uncommon cause of infection in humans and should be considered as a potential pathogenic agent of corneal ulcer. In 2015, Huang et al. [27] reported successful treatment of fungal keratitis due to C. albidos with amphotericin B. In 2014, Liu et al. [28] presented the first case of encephalitis due to C. albidos in an HIV patient. Intravenous fluconazole was applied for him but he died on day three.

In 2015, Ragupathi et al. [29] reported the first case of C. albidos peritonitis in a patient infected with hepatitis C who was undergoing peritoneal dialysis due to renal failure. The patient was treated with amphotericin B; however, infections of non-neoformans cryptococcal species presented a clinical challenge because they are complicated to diagnose and treat.

Cryptococcus albidos has five varieties, namely C. albidos var. aeriys, C. albidos var. albidos, C. albidos var. diffuens, C. albidos var. kaezungii, and C. albidos var. ovalis. One isolate of C. albidos var. kaezungii was obtained from pet shops in the present study.

Cryptococcus uzbekistanensis is a non-capsulated yeast that was first isolated from a desert soil sample from near Bukhara, Uzbekistan, in 1999 by Chernov et al. Afterwards, Fonseca et al. identified that this species causes glossy, smooth, cream to pinkish-cream colonies on yeast mold agar, with soft and butyrous texture. Review of veterinary and medical papers reveals that C. uzbekistanensis has never been isolated from an infection in humans or animals [30] until Powel et al. [31] reported the first case of cryptococcosis due to C. uzbekistanensis from the bone marrow of an immunocompromised patient with pancytopenia. Further, isolating C. uzbekistanensis from dust in US military samples has been reported in the Middle East [32]. We also isolated one case of C. uzbekistanensis from pigeon droppings in pet shops. We also reported an isolate of Filobasidium uniguttulatum from pet shops. Filobasidium uniguttulatum, is a teleomorphic fungus, which was first isolated in 1934 from an infected human nail, and then it was identified as Eutorulopsis uniguttulata [33]. On the basis of physiological and morphological similarities with C. neoformans, Eutorulopsis uniguttulata was renamed to Cryptococcus neoformans var. uniguttulatus, but with less capsule formation [34].

Phylogenetically, F. uniguttulatatum is closer to C. albidos than to C. neoformans [35]. Among environmental strains, one case of Cryptococcus neoformans var. grubii was isolated from pigeon droppings in an attic. Cryptococcus neoformans is subdivided into two variants, that is, C. neoformans var. neoformans (var. D) and C. neoformans var. grubii (var. A). The name “grubii” was chosen in honor of David Gruby, physician and scientist who lived in the 19th-century and first recognized and proved dermatophytosis. Cryptococcus neoformans var. grubii has a cosmopolitan distribution and is a fatal fungal pathogen among immunocompromised population [36].

In 2011, Hedayati et al. [11] isolated five Cryptococcus neoformans isolates from 97 environmental specimens from swallow excreta in Sari, Iran. All the yeasts were identified by colony characteristics on Niger seed agar (Guizotia abyssinica) and CGB medium. Khosravi et al. [12] used the same protocol for the isolation and identification of Cryptococcus species from pigeon (Columba livia) droppings in northern cities of Iran (Rasht, Ramsar, Babol, Sari, and Gorgan) and showed that all the isolates were C. neoformans var neoformans. Using CGB test and RapID Yeast Plus System, Soltani et al. [10] identified 3 out of 120 specimens as Cryptococcus neoformans. Out of 400 pigeon dropping specimens, Agha Kuchak Afshari et al. [37] found 20 (5%) samples positive for C. neoformans by sequence analysis of PCR products of the
D1/D2 regions. In 2017, Dou et al. [38] characterized C. neoformans complex from environment in Beijing, China, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). They determined serotypes and mating types of isolates by specific primers, and restriction fragment length polymorphisms of URA5 (URA5-RFLP) were used for genotyping. In addition, multi-locus sequence typing (MLST) was performed for further identification. They identified 81 (18.6%) isolates of C. neoformans AFLP1/VNI; all the strains were serotype A. However, they did not isolate any other molecular types of C. gattii and C. neoformans strains.

Conclusion

We isolated and presented six species and variants of Cryptococcus and many other yeasts in pigeon droppings and Eucalyptus tree specimens. These potentially pathogenic yeasts can cause fatal fungal infections in immunocompromised individuals. The presence of pigeons and Eucalyptus trees close to some places such as rest homes and hospitals should be considered as a risk factor for this vulnerable population.

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Author’s contribution

R. M. and A. S. designed and supervised the research, A. K. performed the tests, and R. M. wrote and edited the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Financial disclosure

The authors declare no financial interests related to the materials of the study.

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