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Presence of pathogenic cryptococci on trees situated in two recreational areas in South Africa

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ABSTRACT

Knowledge of the environmental prevalence of members of the *Cryptococcus neoformans/Cryptococcus gattii* species complex is important, since cryptococcal infection is acquired from the environment. We determined whether trees located in two South African recreational areas harboured pathogenic cryptococci and compared the isolates to clinical isolates obtained from Western Cape hospitals with molecular typing techniques. The majority of isolates originating from trees in a public park in Cape Town (PPCT) were *C. gattii sensu stricto*, followed by *C. neoformans sensu stricto* genotype AFLP1/VNI. The PPCT trees might be a source of infection, since all genotype AFLP1/VNI isolates from these trees and one clinical isolate belonged to the same sequence type (ST), i.e. ST23. Recombination and basidiospore production might be occurring in PPCT trees that contained *C. gattii s.s.* isolates belonging to both mating types. The presence of *C. gattii s.s.* in PPCT trees might therefore pose a risk to human health.

1. Introduction

Cryptococcosis is a major cause of morbidity and mortality among individuals suffering from HIV/AIDS, especially within sub-Saharan Africa (Walsh and Groll, 1999; Park et al., 2009). Members of the *Cryptococcus neoformans/Cryptococcus gattii* species complex are the main etiological agents of this disease. Considering their importance, an array of molecular typing techniques has been developed to distinguish members of this species complex from each other. These techniques have identified five serotypes and ten major genotypes among members of the *C. neoformans/C. gattii* species complex (Franzot et al., 1999; Meyer et al., 1999; Boekhout et al., 2001; Kwon-Chung et al., 2002; Latouche et al., 2003; Meyer et al., 2003; Hagen et al., 2010). In addition, members of the species complex can be further grouped into different sequence types (STs) using multi-locus sequence typing (MLST) (Meyer et al., 2009). Recently the taxonomy of the *C. neoformans/C. gattii* species complex was revised and it was proposed that this complex consists of at least seven species (Hagen et al., 2015), i.e. *C. neoformans s.s.* (syn. *C. neoformans* var. *grubii*, serotype A, genotypes AFLP1/VNI, AFLP1A/VNB/VNII and AFLPB/VNII), *Cryptococcus deneoformans* (syn. *C. neoformans* var. *neoformans*, serotype D, genotype AFLP2/VNIV), *C. gattii s.s.* (syn. *C. gattii*, serotype B, genotype AFLP4/VGI), *Cryptococcus bacillisporus* (syn. *C. gattii*, serotype C, genotype

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AFLP5/VGIII), *Cryptococcus deuterogattii* (syn. *C. gattii,* serotype B, genotype AFLP6/VGII), *Cryptococcus tetragattii* (syn. *C. gattii,* serotype C, genotype AFLP7/VGIV) and *Cryptococcus decagattii* (syn. *C. gattii,* serotype B, genotype AFLP10). In addition to these seven species, hybrids of *C. neoformans s.s.* × *C. deneoformans* (serotype AD, genotype AFLP3/VNIII) (Boekhout et al., 2001), *C. gattii s.s.* × *C. deneoformans* (serotype BD, genotype AFLP8) (Bovers et al., 2006), *C. gattii s.s.* × *C. neoformans s.s.* (serotype AB, genotype AFLP9) (Bovers et al., 2008) and *C. neoformans s.s.* × *C. deuterogattii* (serotype AB, genotype AFLP11) (Aminnejad et al., 2012) have also been described.

Molecular epidemiology studies conducted across the globe have revealed that *C. neoformans sensu lato* is the main etiological agent of cryptococcosis in HIV/AIDS patients and other immunocompromised individuals (Mitchell and Perfect, 1995; Vilchez et al., 2002; Kiertiburanakul et al., 2006). Yet, these yeasts may also infect immunocompetent patients, especially those residing in East Asia (Chen et al., 2008; Pan et al., 2012). In contrast, *C. gattii sensu lato* is primarily associated with cryptococcosis in immunocompetent individuals (Byrnes et al., 2011), but has also been found to infect immunocompromised patients (Chaturvedi et al., 2005; Litvintseva et al., 2005; Hagen et al., 2012). It is believed that cryptococcal infection is acquired by inhalation of infectious propagules, i.e. basidiospores or desiccated yeast cells, that are released by these pathogenic cryptococci into the environment (Sukroongreung et al., 1998; Velagapudi et al., 2009).

Since infection is acquired from environmental sources, it is not surprising that numerous studies have been conducted globally to isolate representatives of the *C. neoformans*/*C. gattii* species complex from different environmental niches. During these studies, *C. neoformans s.l.* was isolated from pigeon droppings (Chee and Lee, 2005; Baroni et al., 2006; Alves et al., 2016) and other avian excreta (Caicedo et al., 1999; Abegg et al., 2006), as well as from the bark, hollows and decaying wood of different tree species (Lazera et al., 2000; Hiremath et al., 2008). Similarly, it seems that the bark, hollows and decaying wood of various tree species are the primary ecological niche of *C. gattii s.l.* (Lazera et al., 2000; Kidd et al., 2007a; Chowdhary et al., 2011). During some of these studies it was found that samples harbouring pathogenic cryptococci were situated in public places, such as church towers, city parks, provincial parks, public squares, schools and zoological gardens (Caicedo et al., 1999; Lazera et al., 2000; Chee and Lee, 2005; Abegg et al., 2006; Baroni et al., 2006; Kidd et al., 2007a; Alves et al., 2016). It is thus likely that when pathogenic cryptococci are present in public areas, they may pose a threat to public health. This is of particular concern in South Africa, considering that an incidence of 103 cryptococcosis cases per 100 000 HIV-positive individuals were reported by GERMS-SA (The Group for Enteric, Respiratory and Meningeal disease surveillance in South Africa) during 2015 (GERMS-SA, 2015). However, evidence of cryptococcal infection acquired from the environment is scarce in South Africa, since only two studies have reported the presence of pathogenic cryptococci in environmental sources (Botes et al., 2009; Litvintseva et al., 2011).

With the above as background we hypothesized that pathogenic cryptococci may be present on trees located in recreational areas in South Africa and that these cryptococci are genetically related to clinical isolates. These hypotheses were tested by first determining whether pathogenic cryptococci are present within woody debris samples collected from trees situated in two recreational areas in South Africa. Secondly, the isolates obtained from these samples were classified according to their species, genotypes, mating types and STs. Finally, these characteristics were also determined for clinical isolates obtained from hospitals in the Western Cape and compared to that of the environmental isolates.

2. Materials and methods

2.1. Environmental sampling

Seventeen trees situated in a public park in Cape Town (PPCT), South Africa were randomly selected for sampling. A total of 22 woody debris samples were collected from these trees on November 27th, 2007 (1st sampling), as well as on July 2nd, 2008 (2nd sampling). Only 21 samples were obtained on March 4th, 2010 (3rd sampling), since one of the trees was removed by park officials prior to this sampling date. In addition to these 65 samples, a total of 14 woody debris samples were collected from nine randomly selected trees situated on a privately-owned game farm in the Northern Cape (GFNC), South Africa on December 24th, 2007. The environmental sampling did not involve endangered or protected species and no specific permits were required. The manager of the public park and the owner of the game farm gave us permission to sample the different trees. All authorizations were granted as verbal agreements.

2.2. Sample processing and yeast isolation

A ten-fold dilution series of each woody debris sample (1 g) was prepared in sterile physiological saline solution (PSS). Subsequently, 0.1 ml of each dilution was spread plated onto Niger seed agar (Yarrow, 1998) supplemented with 100 mg/l biphenyl (Sigma-Aldrich, St Louis, MO, USA) and 200 mg/l chloramphenicol (Sigma-Aldrich) (NS_{BC} agar) and these plates were incubated at 30 °C for 4 d. However, this growth medium is non-selective and allows the overgrowth of filamentous fungi, which can obstruct the isolation of pathogenic cryptococci and lead to false-negative results (Fortes et al., 2001; Kobayashi et al., 2005). Furthermore, using the above-mentioned standard dilution plate method to enumerate and isolate unicellular microorganisms may lead to false negative results when the number of the target organism is below the detection limit of this method. Therefore, an enrichment technique was employed and 1 g of each woody debris sample was also placed in a 250 ml conical flask containing 30 ml Niger seed broth (Yarrow, 1998) supplemented with 200 mg/l chloramphenicol (Sigma-Aldrich) (NS_C broth). These flasks were incubated at 30 °C for 4 d on a G53 rotary shaker (New Brunswick Scientific Co. Inc., Edison, NJ, USA), set to 100 revolutions per minute (rpm). During this step the sporulation of filamentous fungi is inhibited, while the filamentous growth is aggregated into pellets, which allow the yeasts to outgrow the filamentous fungi (Wickerham, 1951). To separate the yeasts from the fungi, 1 ml of the culture present in each flask was filtered through sterile glass wool (Yarrow, 1998) into flasks containing fresh NS_C broth. The new set of flasks was incubated at 30 °C for 4 d on a rotary shaker (100 rpm), thereafter the filtering step was repeated. Following incubation under the same culturing conditions, a ten-fold dilution series was prepared of each culture present in the third set of flasks in sterile PSS. Subsequently, 0.1 ml of each dilution was spread plated onto NS_{BC} agar and these plates were incubated at 30 °C for 4 d.

After incubation, brown pigmented colonies were randomly isolated from the NS_{BC} agar plates using a modification of the Harrison's disc method (Harrigan and McCance, 1976) and were re-cultured on NS_{BC} agar to confirm the production of melanin. The obtained yeast isolates were then purified by successive inoculation and incubation at 30 °C on yeast extract-malt extract (YM) agar (Yarrow, 1998) supplemented with 200 mg/l chloramphenicol (Sigma-Aldrich). Pure isolates were maintained on YM agar slants at 26 °C and were stored at −80 °C in 15% (v/v) glycerol, as part of the culture collection of the Department of Microbiology, Stellenbosch University, South Africa. The environmental isolates were