Environmental Survey for *Cryptococcus gattii* in an Israeli Zoo Populated with Animals Originating from Australia

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ABSTRACT

Cryptococcus gattii is an emerging human and animal fungal pathogen. The first isolation of *C. gattii* from an environmental source was from organic material adjacent to eucalyptus trees (*Eucalyptus camaldulensis*) in Australia but subsequently the yeast was isolated from decaying wood of other tree species. It has been suggested that a unique infectious cycle, between koalas (*Phascolarctos cinereus*) and the eucalyptus trees they live and feed on, facilitates the persistence of the fungus in the environment. Since *C. gattii* has not been reported in Israel, a zoo populated with animals originating from Australia, including koalas fed with eucalyptus leaves, was deemed a suitable place to conduct the first environmental survey for the presence of this microorganism in this country. The survey was conducted in two seasons (winter and summer). Environmental samples were collected from different sites inside and around the koala's cage. Fur and nail samples and nasal swabs of the koalas were cultured on dopamine agar and sera were tested for the presence of specific cryptococcal antigen. All environmental samples and samples taken from the koalas were negative, with no evidence of *Cryptococcus* spp. colonies. Serum samples were negative for cryptococcal antigen. Additional environmental surveys in Israel, focusing on different ecological niches and especially on a larger variety of tree species are suggested.

Keywords: *Cryptococcus gattii*; Koala (*Phascolarctos cinereus*); Eucalyptus; Israel; Zoo

INTRODUCTION

The genus *Cryptococcus* comprises 37 basidiomycetous yeast species, among which *Cryptococcus neoformans* and *Cryptococcus gattii*, are the main human and animal pathogens (1). While *C. neoformans* usually requires a status of immunosuppression to infect a patient, *C. gattii* does meet this requirement (2). *C. gattii* is an emerging pathogen, first reported in 1970 in the Congo (3) as C. *neoformans* var. *gattii*. Subsequently it was reclassified as a distinct species as *C. gattii* (4).

While originally limited to tropical and subtropical environments (5, 6), *C. gattii* has been involved since 1999 in an ongoing outbreak that started in Vancouver island in Canada and then spreading through the Pacific Northwest of the United States, albeit with some variability of the genotype involved (6). An interesting hypothesis as to the source of these highly pathogenic strains from the Amazon basin has been proposed (7).

Both pathogenic *C. neoformans and C. gattii* are characterized by being surrounded by a capsule (8). *In vitro* they produce of melanin on media containing diphenol compounds such as 3,4-dihydroxyphenylalanine (L-DOPA), resulting in dark tan colonies, which is considered as an enhancing factor

of the fungus' ability to survive in the environment (9) and as a virulence factor (10, 11). Two teleomorphs *Filobasidiella neoformans* and *Filobasidiella gattii* have been described for *C. neoformans* and *C. gattii*, respectively. Canavanine-glycine bromthymol blue (CGB) agar is successfully used to differentiate between the two species (12).

The source of infection with *C. gattii* is mostly environmental. The importance of environmental "hot spots" was demonstrated first in Australia by Ellis and Pfeiffer (13), showing the fungus to be associated with organic material around eucalyptus (*Eucalyptus camaldulensis*) trees. Subsequently such associations were shown in other niches as well (14, 15, 16, 17, 18). Moreover, during the blooming period of the eucalyptus trees, the environmental dissemination of *C. gattii* has been found to be increased (13).

Direct transmission between humans or animals is rare (19, 20). Clinically, infection may vary from asymptomatic carrier states to disseminated, lethal ones (1, 21).

C. gattii was has been found in a variety of other animals, wild and domestic (22, 23), showing disparate clinical signs (6). Being endemic in Australia, the interaction between *C. gattii*, koalas (*Phascolarctos cinereus*) and the eucalyptus trees they live and feed on has generated special interest. Due to their habitat and feeding pattern, these animals are at high risk of contracting *C. gattii*, resulting in clinical infections or carrier states where the yeast may colonize the upper respiratory tract and skin (24). The prevalence of carrier animals was found to be directly proportional to the yeast's presence in the environment, possibly indicating a unique infectious cycle that facilitates the persistence of the fungus in the environment. Antigenemia, sometimes transitory, was found in carrier animals (25).

A survey was undertaken to study the possible presence of the zoonotic agent *C. gatti* in Israel. For this purpose, a zoo populated with animals originating from Australia, including koalas fed with eucalyptus leaves was surveyed.

MATERIALS AND METHODS

Environment and animals

The survey was conducted over the years 2010-2011 in a zoo ("Gan-garoo"), with an area of about 1.2 hectares, located in the north of Israel. It was populated by various animals originating from Australia, some of them, notably different species of kangaroos, roaming freely. The zoo's flora's composition has been devised so as to mimic the one the animals were used to in their original environment and was based mostly on eucalyptus trees. At the time of the survey there were 3 koalas in the zoo. A male and a female kept in one cage and an additional male, separately, in a second cage. The koalas had no direct contact with the visitors. The details of the koalas are presented in Table 1.

Table 1: Details of the koalas present at the zoo at the time of the survey.

Name	Sex	Subspecies	Origin	Birth	At zoo since
Mindy	Female	*Victor*	Kyabram	1998	2002
Didge	Male	*Cinereus*	Sydney	2002	2002
Milo	Male	*Victor*	Ballarat	2006	2006

In the koalas' cages, dry eucalyptus branches, serving the animals' activities were replaced once every few months, Fresh eucalyptus leaves of various species (*E. camaldulensis, E. viminalis, E. robusta, E.maculata*) were grown at a designated orchard and were kept refrigerated after harvesting for no more than 2 days before feeding them to the koalas. The cages' floor was mostly covered by concrete and washed daily.

Sampling

To assess an impact of the weather on the presence of *C. gattii*, the cages' environment was sampled twice, once in January 2010 and then in September 2011. In the first sampling cycle, 37 sterile swabs were inserted into nooks of the dry eucalyptus branches and recesses on the cages' floor. In the event that resampling would be necessary, the sites were marked (Figure 1). In addition eucalyptus leave samples from the animals' feed were taken. In the second sampling cycle (September 2011), 25 environmental swabs were taken as previously described. In addition, fresh eucalyptus leaves from the trees adjacent to the cages but not in direct contact with the koalas, were sampled, about 30 on each occasion.

In April 2010, swabs from the coat, nails and nares and blood samples for cryptococcal antigenemia testing of two koalas (Mindy and Didge) were taken by the zoo personnel (Figure 2). Sampling of the third koala, Milo, was not undertaken as he was too aggressive to be manipulated without anesthesia.

Laboratory examinations

Environmental swabs from the first cycle were inoculated onto dopamine agar (26, 27) *in situ* after being transported

Figure 1: Sampling spots on dry eucalyptus branches.

under refrigeration to the laboratory. Swabs from the second cycle were inoculated in the laboratory, onto Niger Seed agar (28). Swabs from the koalas were inoculated *in situ* directly after suspension in 5 ml sterile saline onto dopamine agar. Leaves were transported to the laboratory, where the leaves were immerged in 400 ml sterile saline and shaken continuously, at 100 rpm, at room temperature, for 7 days. Subsequently the saline was distributed to 8x50 ml tubes and centrifuged at 500 x g for 15 minutes. The supernatant was inoculated onto dopamine agar (the first cycle) or Niger Seed agar (the second cycle). The pellet was inoculated onto the same media undiluted and suspended in 25 ml or 45 ml saline. All media were incubated at 30°C for 14 days and examined daily.

The koalas' sera were examined for Cryptococcal antigen using a commercial kit (Cryptococcal Antigen Latex Agglutination System (CALAS®), Meridian Bioscience, USA).

Figure 2: Sampling the koalas.

RESULTS

All samples (environmental and those from the koalas) were negative for *Cryptococcus* spp. in general and *C. gattii* in particular.

DISCUSSION

This unique survey, aimed at assessing the presence of *C. gattii* in an Israeli zoo populated with animals originating from Australia, is the first one of its kind performed in Israel. To the best of our knowledge *C. gattii* has not been isolated in this country, neither from humans nor from animals or the environment, and thus the likelihood of finding it in the zoo was low. However, being a sanctuary of animals originating from Australia where the fungus is endemic, the surveyed zoo was especially suitable to perform this first survey. Moreover, considering the zoonotic potential of *C. gattii* and the large number of visitors at the zoo, especially children, it was important to exclude its presence, at least at levels detectable by the methods employed.

The fact that we did not find *C. gattii* in our survey may be the result of its absence in the zoo or due to lack of a more extensive sampling. The number of animals present at the time of the survey was very low and all koalas were thoroughly examined before being exported from Australia. Even in the case of a carrier animal being introduced, the likelihood of the fungus completing its life cycle is low due to the fact that there is no contact between the koalas and live eucalyptus trees.

The isolation of *C. gattii* from the environment is dif-

ficult and the rate of positive samples is relatively low: in the Australian Northern Territories, for instance, it took 2000 samples to isolate the fungus (13). Similarly, in India only 3 samples out of 696 (0.4%) (28) were positive, whereas in Brazil the corresponding numbers were 1 out of 260 (0.38%) (29). In the Middle East and Mediterranean Basin several attempts have been made to isolate *C. gattii* from the environment in Turkey, Jordan, Egypt, Italy and Spain (18, 31, 32, 33). It was isolated in Egypt from 3 samples out of 245 (1.2%), in Italy from 6 out of 255 samples (2.35%) and in Spain from 14 samples out of 479 tested (2.9%). Interestingly, in Spain *C. gattii* was found in association with carob trees (*Ceratonia siliqua*) (18, 34), thus indicating that future environmental surveys in Israel should include additional tree species as well.

The above-mentioned reports showed that *C. gattii* may be present in our region and that the number of samples we examined provided a fair chance of detecting the yeast, were it present. Moreover, the presence of *C. gattii* in the environment is usually linked to human and/or animal cases of infection (18, 29, 32, 33,35). The fact that the fungus was thus far not isolated in Israel further substantiates the likelihood that its has a very low prevalence or is absent in Israel.

Nevertheless, its presence in the Middle East and Mediterranean Basin indicates that this environment is suitable as a niche and consequently it may be introduced in the future, for example by marine mammals, as has been previously reported (36). Such animals that are stranded on Israeli beaches are routinely inspected for infectious organims, including fungi (37). Moreover, human and veterinary mycological laboratories must be alert to identify *C. gattii* should it emerge in Israel.

DECLARATION OF CONFLICTING INTERESTS

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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