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Microbe Hunting: A Curious Case of Cryptococcus

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Abstract : *C. neoformans*-associated cryptococcosis is primarily a disease of immunocompromised persons, has a world-wide distribution, and is often spread by pigeons in the urban environment. In contrast, *C. gattii* causes infection in normal hosts, has only been described in tropical and semi-tropical areas of the world, and has a unique niche in river gum Eucalyptus trees. Cryptococcosis is acquired through inhalation of the yeast propagules from the environment. *C. gattii* has been identified as the cause of an emerging infectious disease centered on Vancouver Island, British Columbia, Canada. No cases of *C. gattii*-disease were diagnosed prior to 1999; the current incidence rate is 36 cases per million population. A search was initiated in 2001 to find the ecological niche of this basidiomycetous yeast. *C. gattii* was found in the environment in treed areas of Vancouver Island. The highest percentage of colonized-tree clusters were found around central Vancouver Island, with decreasing rates of colonization to the north and south. Climate, soil and vegetation cover of this area, called the Coastal Douglas fir biogeoclimatic zone, is unique to British Columbia and Canada. The concentration of airborne *C. gattii* was highest in the dry summer months, and lowest during late fall, winter, and early spring, months which have heavy rainfall. The study of the emerging colonization of this organism and subsequent cases of environmentally acquired disease will be informative in planning public health management of new routes of exposure to exotic agents in areas impacted by changing climate and land use patterns.

Keywords : Cryptococcus, yeast, ecological niche, Vancouver Island

Cryptococcosis is an infection associated with an encapsulated, basidiomycetous yeast *Cryptococcus neoformans*. The route of entry for this organism is through the lungs, with possible systemic spread via the circulatory system to the brain and meninges. There are four cryptococcal serogroups associated with disease in humans and animals, distinguished by capsular polysaccharide antigens. *Cryptococcus neoformans*: variety *grubii* (serotype A), variety *neoformans* (serotype D), and variety *gattii* (serotypes B and C) (Franzot *et al.*, 1999). *C. neoformans* variety *gattii* has recently been elevated to species status, *C. gattii*.

C. neoformans var. *grubii* and var. *neoformans* have a world-wide distribution, and are particularly associated with soil and weathered bird droppings. In contrast, *C. gattii* (CG) is not associated with bird excrement, is primarily found in tropical and subtropical climates, and has a restricted environmental niche associated with specific tree species. (Ellis and Pfiffer, 1990).

Ellis and Pfeiffer theorize that, as a basidiomycete, CG requires an association with a tree in order to become pathogenic to mammals. In Australia, CG has been found to be associated with five species of Eucalypts, *Eucalyptus camaldulensis*, *E. tereticornis*, *E. blakelyi*, *E. gomphocephala*, and *E. rudis*. Eucalypts, although originally native to Australia, now have a world-wide distribution. CG has been found associated with imported eucalypts in India, California, Brazil, and Egypt. In addition, in Brazil and Columbia, where eucalypts have been naturalized, native trees have been shown to harbour CG (Callejas *et al.*, 1998; Montenegro *et al.*, 2000).

In British Columbia, Canada, since the beginning of 1999, there have been 120 confirmed cases of

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cryptococcal mycoses associated with CG in humans, including 4 fatalities (data from British Columbia Centre for Disease Control), and over 200 cases in animal pets in BC (data from Central Laboratory for Veterinarians).

What is remarkable about the BC outbreak of *C. gattii*-cryptococcosis is that all of the cases have been residents of, or visitors to, a narrow area along the eastern coast of Vancouver Island, BC, from the tip of the island in the south (Victoria) to Courtenay on the north-central island as illustrated in Fig. 1. Of the first 38 human cases, 58% were male with a mean age of 59.7 years (range 20-82); 36 cases (95%) were Caucasian. Ten cases (26%) presented with meningitis, the remainder presented with respiratory symptoms. Cultures recovered from cases of cryptococcosis associated with the outbreak were typed as serogroup B, which is specific to CG (Bartlett *et al.*, 2003)

This was the first reported *outbreak* of CVG in Canada, or indeed, the world. Where infection with CG is endemic, for example, Australia, the incidence of cryptococcosis ranges from 1.8-4.7 per million between the southern and northern states (Sorrell, 2001). However, the overall incidence of cryptococcosis in immunocompenent individuals has been estimated at 0.2 per million population per year (Kwon-Chung *et al.*, 1984). The population of Vancouver Island is approximately 720,000, consequently, even if the organism were endemic, one would expect a maximum of 0.15 cases of cryptococcal disease annually.

Environmental *Cryptococcus*

The area along the eastern side of Vancouver Island where animal and human cases have clustered is defined by soil and plant cover as a unique biogeoclimatic zone called the Coastal Douglas-fir zone. This zone, defined by geology of soil and plant cover, is characterized by very dry summers and very mild, wet winters compared to BC or Vancouver Island averages.

Eucalyptus trees are not native to BC, although they have been introduced as ornamentals. The

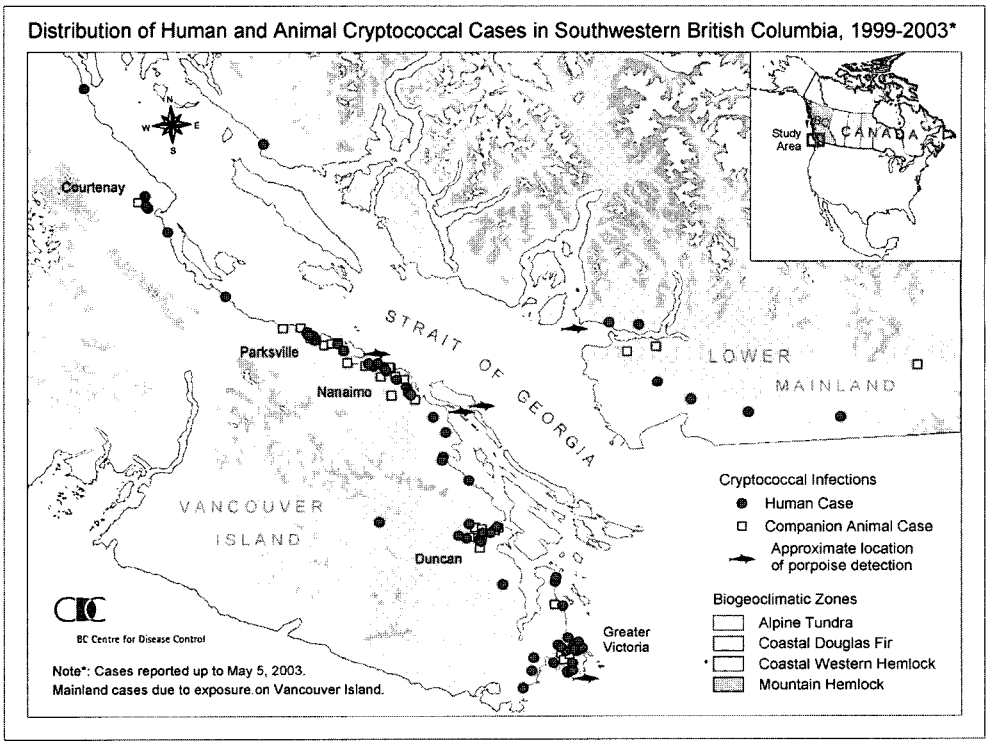

Fig. 1. Distribution of cases of *C. gattii*-cryptococcosis (map created by Sunny Mak).

eastern coast of Vancouver Island falls within USDA zone 8 (a horticultural guidance to the "cold hardiness" of plants). Some 19 species of eucalypts are hardy to zone 8, and are available commercially in BC. However, *E. camaldulensis* and *E. tereticornis,* the primary ecological niches of CG in their native Australia, are not tolerant to zone 8, and have never been commercially propagated in BC.

The search for environmental sources of CG commenced in BC at the end of 2001. The first positive environmental cultures were identified in 2002 in Douglas Fir (*Pseudotsuga menziesii*) and Red Alder (*Alnus rubra*), trees native to BC.. Quantitative air samples were taken under the culture positive trees, and a high concentration of airborne CG were recovered (1080 CFU/m^3) using an Andersen six-stage sampler. In this sample, about 8% of the CG were of a size range that could enter the deep lung (0.65-3.3 μm) (Bartlett *et al.*, 2005).

Materials and Methods

Sampling Sites

The search strategy used to locate environmental sources of *C. gattii* was to sample trees in the environs of cases (human or animal) matching the criteria of outbreak-associated cryptococcosis. No attempt at randomization was made during this phase of the study due to the vast areas of treed areas in BC and the difficulty in reaching areas without roads, etc. Therefore, the trees sampled were in areas where members of the public might be exposed, for example, public parks, wooded trails, landscaped gardens, etc. Choosing sampling sites near animal cases was particularly useful because companion animals, particularly cats, do not often travel away from their residence as do humans.

Microbiological Sampling

(1) Swab samples were taken using Starswab II™ swabs, transported in clear Amies media to screen for CG colonization of tree bark; **(2) topsoil** (including leaf debris) was collected from beneath the foliage canopy into a ziplock bag; and **(3) air** samples were taken using a Reuter Centrifugal Sampler (RCS) or Andersen six-stage sampler directly onto Staib agar. Swabs, soil and air samples were returned to the Environmental Bioaerosol Exposure Laboratory at the University of British Columbia. Swabs and soil were plated onto Staib agar (formulation per litre: Niger seed extract, 200 m*l*; glucose 1 g; creatinine 0.78 g; potassium phosphate 1 g; chloramphenicol, 0.4 g; agar, 15 g) and incubated at 30°C for 48-96 hr. Air samples were similarly incubated.

Presumptive positive cultures were transferred onto Canavanine-Glycine-Bromothymol Blue (CGB) agar. Cultures which formed melanin on Staib agar, hydrolyzed glycine in the presence of L-canavanine on CGB agar, and conformed morphologically to *Cryptococcus* were serotyped using purified antibodies to capsular antigens (Crypto Check, Iatron Laboratories).

Molecular fingerprinting was performed on human, animal, and environmental isolates using polymerase chain reaction (PCR)-URA5 restriction fragment length polymorphism (RFLP) (Meyer *et al.*, 1999).

Results

PCR-fingerprints

Clinical cultures from immunocompetent humans and animals, and environmental isolates all belonged to Serogroup B. PCR-RFLP analysis of clinical and environmental isolates revealed two genetic variants, VGII (93%) and VGI (7%). Fig. 2 illustrates the BC-associated *C. gattii* (genotype VGII). Two variants of VGII (VGIIa and VGIIb) have been found in human and environmental cultures. Further work is planned to determine if these variants are associated with virulence factors.

Swab Samples

Table 1 lists the range of tree species that have been tested in British Columbia for the colonization of *C. gattii.* In this study, with the exception of Hemlock, native trees were found to have higher rates of colonization (5-22%) than did imported or ornamental trees (0-4%). No Eucalyptus trees were found to be colonized.

However, we found that colonized trees were not evenly distributed in BC. Rather, certain geographic

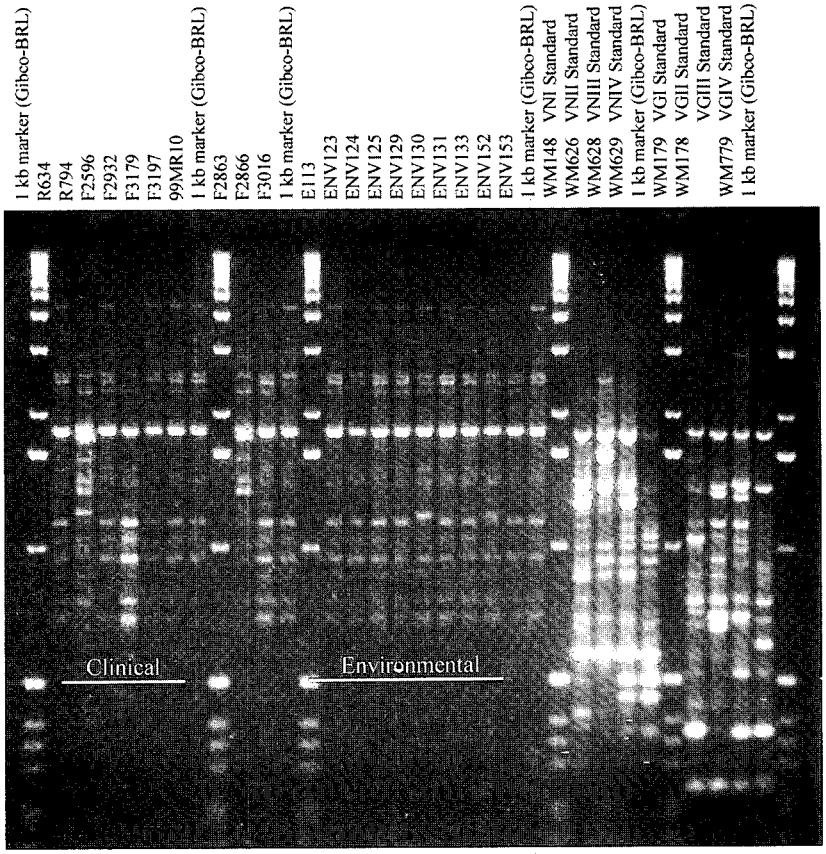

Fig. 2. PCR-fingerprint of clinical and environmental culture isolates of *C. gattii*.

Table 1. *C. gattii* colonization patterns in sampled trees in BC

Tree	n	Swab		Percent positive
		Positive	Negative	
Native coniferous/evergreen				
Arbutus (*A. menziesii*)	127	15	112	22
Cedar (*Thuja*)	329	40	289	22
Douglas fir (*Pseudotsuga*)	915	95	820	10
Hemlock (*Tsuga*)	41	0	41	0
Pine (*Pinus*)	81	4	77	5
Non-native evergreen				
Eucalyptus (*Eucalyptus*)	25	0	25	0
Spruce (*Picea*)	41	2	39	5
Deciduous				
Alder (*Alnus*)	252	27	225	11
Garry Oak (*Quercus*)	113	16	97	14
Maple (*Acer*)	228	12	216	5
Other:				
Other trees, non-native	159	7	152	4
Shrubs	16	0	16	0

Table 2. Trees sampled for *C. gattii*-colonization listed by location

Location	n	Swab		Percent positive
		Positive	Negative	
East coast Vancouver Island				
Victoria	488	15	473	3
Duncan	207	14	193	7
Ladysmith	34	0	34	0
Nanaimo	324	3	321	1
Parksville	455	135	320	30
Courtenay	261	16	245	6
Campbell River	24	0	24	0
West of Parksville				
Cameron Lake	193	14	179	7
Port Alberni	60	11	49	18
West coast of Vancouver Island	23	0	23	0
Mainland BC	270	0	270	0

areas had clusters of positive trees, while in other areas no colonized trees were found, as listed in Table 2.

To date, no trees have been found to be colonized with *C. gattii* on the Mainland of BC or the West Coast of Vancouver Island. The east coast of central Vancouver Island has the highest rate of positive samples, with lower percentages of colonization to the north and south. Port Alberni, with a high percentage of colonized trees, is about 40 km to the west of Parksville, and is connected by a heavily traveled road which runs through a river valley.

Air Samples

Air samples were taken in the locations where trees were sampled. Table 3 lists concentrations of airborne propagules by area. A significant number of samples taken were negative, and the analysis was repeated on the positive air samples to more accurately reflect the exposures which may occur near culture-positive trees.

Table 3. Airborne concentrations of *C. gattii* by location in BC

Location	n	All air samples		Air samples above LOD[d]	Positive air samples
		GM[a] CFU[b]/m^3 (GSD[c])	[range]	n	GM CFU/m^3 (GSD)
Victoria	49	2 (4.54)	[0-871]	10	30 (3.98)
Duncan	45	0.5 (6.82)	[0-1514]	16	30 (6.19)
Ladysmith	3	5 (5.02)	[0-25]	2	12 (2.74)
Nanaimo	17	2 (2.92)	[0-25]	6	8 (1.87)
Parksville	69	6 (9.42)	[0-2089]	32	47 (5.45)
Courtenay	50	2 (3.86)	[0-1202]	8	20 (7.70)
Cameron Lake	9	4 (9.07)	[0-182]	3	51 (7.47)
Port Alberni	11	2 (3.54)	[0-42]	2	40 (2.74)
Gulf Islands	8	3 (6.12)	[0-50]	2	50 (0)
Mainland	55	1 (2.09)	[0-38]	5	11 (2.66)

[a]Geometric mean of lognormally distributed data.
[b]CFU = Colony forming units.
[c]Geometric standard deviation of lognormally distributed data.
[d]LOD = Limit of detection = 5 CFU/m^3.

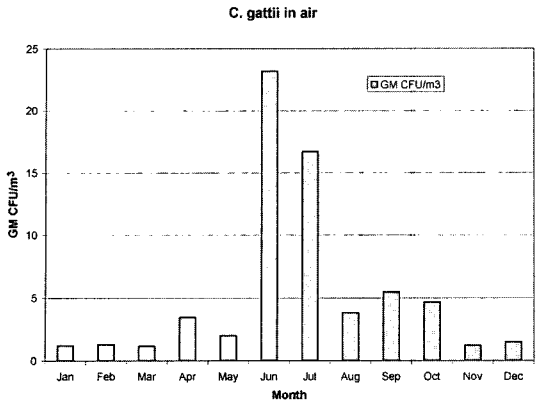

Fig. 3. Airborne concentration of *C. gattii* by month.

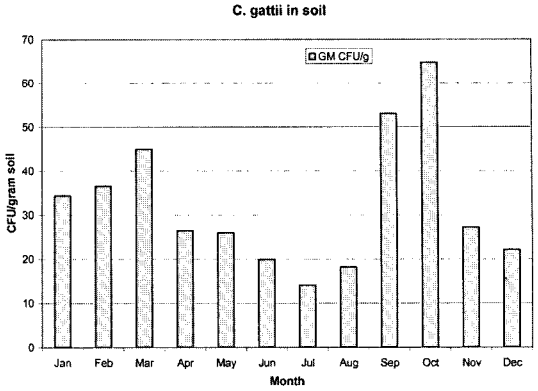

Fig. 4. Soil concentration of *C. gattii* by month.

There was a very strong influence of season on the recovery of airborne propagules of *C. gattii* as illustrated in Fig. 3. The highest airborne concentrations of propagules were detected in the summer months, when the day and nighttime temperatures were highest, and the amount of precipitation the lowest. In general, the concentration of the organism was much lower in air once the fall and winter rains began, and on sampling days when rain was falling, no organisms were recovered.

Soil Samples

Soil samples were taken in areas where trees were sampled. Table 4 lists concentrations of *C. gattii* in soil. The gradient of concentrations follow closely with the clustering of positive trees, with higher concentrations found in central Vancouver Island, with lower concentrations to the north or south. In some cases, soil samples were positive, while the swab samples were negative. This may be a function of season, as the soil samples were not influenced to the same extent by season as were the air and swab samples as seen in Fig. 4.

The organism can be spread by air currents, the movement of wood products, soil, and carriage on the soles of shoes or wheel wells of cars. Fig. 5 is the summary of an experiment in which trees along an east-west corridor road were sampled every 500 m. This road has high traffic volume in the summer months because there are several popular forested parks along its route. The trees in this series which were culture-positive for *C. gattii* were near parking lots or pull-outs, suggesting that vehicles may be a source of transport of *Cryptococcus*. However, the organism may be transient in bio-

Table 4. Soil concentrations of *C. gattii* by geographic area in BC

Location	n	All soil samples		Soil samples n	Positive soil samples
		GM CFU/g (GSD)	[range]		GM CFU/g (GSD)
Victoria	188	1.4 (4.54)	[0-2042]	13	127 (4.32)
Duncan	117	2.7 (12.4)	[0-36308]	18	706 (8.75)
Ladysmith	11	2.2 (13.8)	[0-6026]	1	6026 (-)
Nanaimo	121	1.5 (3.80)	[0-708]	10	107 (3.39)
Parksville	138	5.7 (13.5)	[0-29512]	49	134 (6.46)
Courtenay	153	1.6 (5.26)	[0-4365]	13	253 (8.43)
Cameron Lake	86	1.7 (6.21)	[0-10471]	8	366 (8.16)
Port Alberni	49	2.2 (5.39)	[0-537]	10	49 (3.77)
Gulf Islands	122	4.9 (22.4)	[0-194985]	30	688 (14.3)
Mainland	122	0	[0]	0	0 (-)

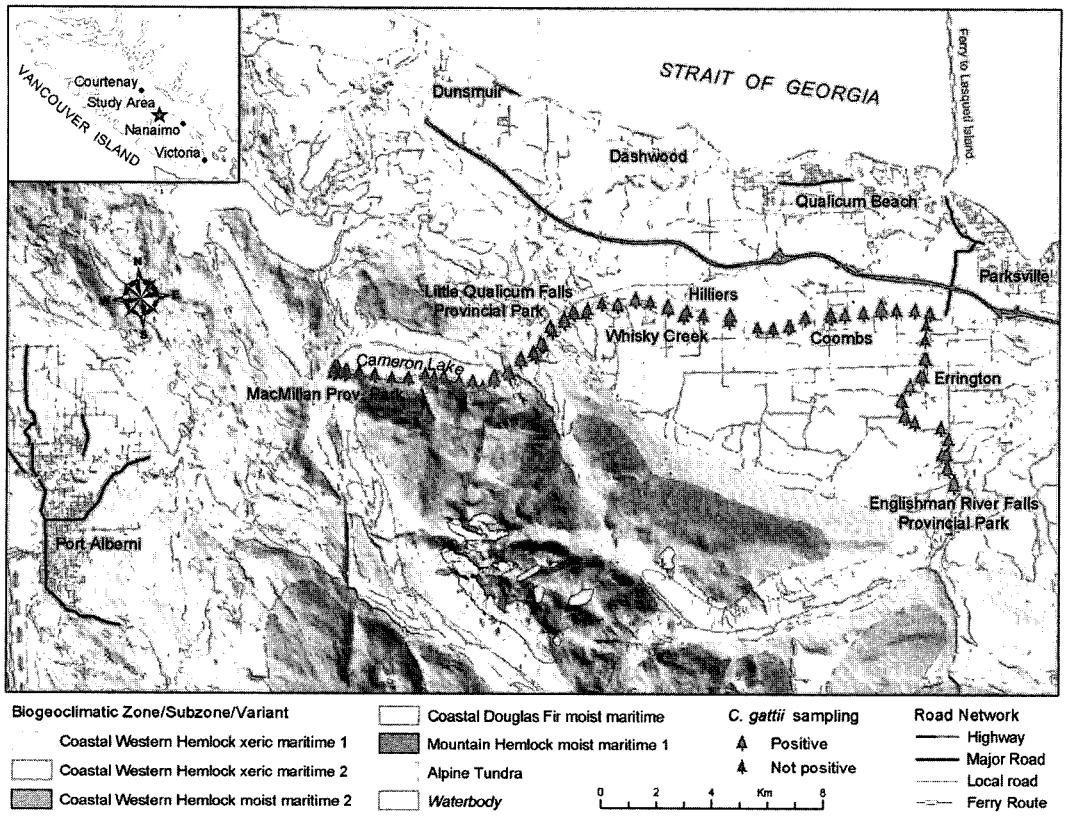

Fig. 5. Possible transfer of *C. gattii* from colonized trees in the Coastal Douglas fir zone to other xeric biogeoclimatic zones.

geoclimatic zones that are not favorable to successful colonization.

Summary

This is the first report of a stable ecological niche for the tropical yeast, *Cryptococcus gattii* in a temperate climate zone. *C. gattii* has been found in a wide variety of trees native to the Coastal Douglas fir biogeoclimatic zone in British Columbia. Cases of cryptococcosis caused by *C. gattii* first appeared in late 1999. The incidence rate of infection is low, 36 cases per million population, but the disease is potentially fatal without proper treatment. The use of geographic data identifying areas where human and animal cases resided allowed recovery of the organism from the environment. Although it is not known how *C. gattii* was brought to Vancouver Island, subsequent spread of the organism may be through human intervention by transport of wood products or soil.

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