

THE EMERGENCE OF *CRYPTOCOCCUS GATTII*
IN BRITISH COLUMBIA:
VETERINARY ASPECTS

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Abstract

A series of presumed or confirmed *Cryptococcus gattii* cases diagnosed between 1999 and 2003 was compiled through review of records from veterinary laboratories and human diagnostic services. There was a continual increase in the annual number of animal, but not human, cases diagnosed; no seasonality was observed. Animal cases exceeded human cases by almost 75% even though it was hypothesized that animal cases are more likely to go undiagnosed or unreported when compared to humans. Animal cryptococcosis cases were identified on Vancouver Island prior to 1999 suggesting the organism may have emerged in the region prior to its identification as a causative agent for human disease; therefore animals may serve as a good sentinel for human cryptococcosis infection.

There were 50% more feline than canine cases and disease appeared more commonly in middle aged cats and younger dogs. There was no sex predilection for either species. The primary system involved was most commonly respiratory, followed by central nervous system (CNS) in both cats and dogs. There was a higher proportion of CNS disease in dogs relative to cats, and cats were much more likely to have subcutaneous or dermal masses relative to dogs. Multivariate survival analysis identified only the presence of neurological symptoms as a statistically significant predictor of mortality; those animals exhibiting CNS symptoms were over four times more likely to die than those never showing neural signs. A case-control study identified host and environmental risk factors for clinical *C. gattii* infection in dogs and cats suggesting that where an infectious agent is not uniformly distributed, individual risk increases when the organism

is re-distributed through large scale environmental disturbance, or when the animal has increased opportunities for exposure through travel or activity level.

Serum samples and material for fungal culture were collected from dogs, cats, horses and terrestrial mammal species residing within the region where clinical cases had been diagnosed. Nasal colonization was identified in squirrels (*Sciurus carolinensis*), horses, dogs and cats. Most of the animals sampled had no signs of systemic infection however asymptomatic infection, defined as the presence of cryptococcal antigen in the bloodstream in the absence of clinical symptoms, was identified in a small number of dogs and cats. Fourteen months of follow-up testing of asymptomatic animals revealed that animals can progress to clinical disease, remain sub-clinically infected, or clear the organism.

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1. Introduction and Literature Review

1.1. Introduction

The term ‘emerging infectious disease’ (EID) is used to describe the expansion of a known pathogen to new host species and/or geographic range, or recent identification of a new infectious agent. Emerging infections have been well documented in human medicine (1) and are increasingly identified in domestic and wild animals (2, 3) as well as agricultural and wild plant species. Emerging infectious diseases may impact populations locally, regionally or globally and effects vary according to relevant host, agent and environment interactions.

Changes in human ecology are a central force influencing EIDs; the emergence of pathogens within different cohorts of biota is fundamentally driven by different forms of environmental anthropogenic change (2). Causal factors implicated in emergence include changes in human behavior patterns, social organization, demographics, movement, industry and land use in conjunction with microbial adaptation that disrupts the host parasite relationship equilibrium such that the parasite is favored (2, 4).

In 2001 the public health authorities and veterinary community of southwestern British Columbia, Canada recognized an increased incidence of human and animal cryptococcosis (5). The cases were largely restricted to Vancouver Island and most isolates were *C. gattii* serotype B; a species classically restricted to tropical and sub-tropical regions of the world. The appearance of *C. gattii* in Canada constitutes an EID as the known pathogen has surfaced in a new geographic region.

1.2. *Cryptococcus* spp. and cryptococcosis

1.2.1. Taxonomy

Cryptococcus spp. are environmental fungi of the phylum Basidiomycota, class Heterobasidiomycetes, order Filobasidiales, family Filobasidiaceae (6). The genus *Cryptococcus* includes over 37 species however only *C. neoformans* was commonly considered to be pathogenic. There were previously three recognized varieties of *Cryptococcus neoformans*: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *gattii* (serotypes B and C) as well as a hybrid of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (serotype AD) (7-9). Serotypes are based on the antigenicity of the capsular polysaccharides. Recently proposed changes to the nomenclature suggest that *C. neoformans* should be divided into two distinct species including *C. neoformans* (serotypes A, D and AD) and *C. gattii* (serotypes B and C) based on molecular and mating type characteristics (10). This nomenclature is now widely accepted and will be used here.

Advances in DNA typing methods have led to the identification of eight molecular types based on polymerase chain reaction (PCR) fingerprinting, random amplified polymorphic DNA analysis and restriction fragment length polymorphism (RFLP) (11-13). Serotypes are consistent with the molecular types of *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) where serotype A is made up of molecular types VNI and VNII and serotype D equates to VNIV. Serotype AD, the hybrid of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*, corresponds to VNIII. In contrast *C. gattii* is comprised of 4 different molecular types, VGI, VGII, VGIII, and VGIV, which do not correspond well with the delineations of serotypes B and C (14).

1.2.2. Ecology and Global Distribution of *Cryptococcus* spp.

1.2.2.1. *Cryptococcus neoformans*

The ecology of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* are quite similar; as *C. neoformans* var. *grubii* was only proposed as a distinct variety, separate from *C. neoformans* var. *neoformans*, in 1999 it is often difficult to discern differing distributions of the two varieties in the literature (15). Historically both varieties of *C. neoformans* were thought to be associated with avian excreta, particularly that of pigeons (16-22). It was hypothesized that birds feeding on an unidentified host plant could carry the organism in their gastrointestinal tract and act as a vector by dispersing yeast cells in their feces. The birds were thought to not be clinically affected because their body temperature was above that required for replication of the fungi (23, 24). This hypothesis was challenged by the isolation of both varieties from living and decaying vegetation and domestic dust worldwide (20, 21, 25, 26). Given that avian excreta is rich in creatinine and other chemical constituents that promote fungal replication, it is likely that the environmental niche of the fungus is vegetation but that it is easily isolated from avian excreta because it provides a good media for growth (21, 24).

C. neoformans var. *grubii* has a global distribution and is the most common cause of human fungal meningitis in immunocompromised hosts worldwide (22, 27-32). *C. neoformans* var. *neoformans* (serotypes D and AD) is less commonly recovered from the environment or clinical cases and appear to be more prevalent in Europe than other parts of the world (32, 33).

1.2.2.2 *Cryptococcus gattii*

Cryptococcus gattii has classically been restricted to the tropics and sub tropics of the world (6, 32). The first environmental isolation of the organism was from eucalyptus trees (*Eucalyptus camaldulensis* and *E. tereticornis*) in Australia (34, 35). Subsequently *C. gattii* has been recovered from material associated with eucalypt species in many other parts of the world including California, India and Brazil (20, 28, 36, 37) and in non-eucalypt tree species from tropical and subtropical areas worldwide (28, 38-40). Most environmental isolates of *C. gattii* have been serotype B, however there are reports of serotype C isolated from almond trees (*Terminallia catappa*) in Columbia and vegetation in southern California (37,40). It has been proposed that dispersal of infectious propagules, the asexual budding yeast or the sexual basidiospores, is linked to the flowering of the eucalypt trees as airborne organisms had, prior to the collection of Canadian environmental isolates, only been detected under a tree in flower (23).

Some studies suggest that alternative environmental sources of *C. gattii* have yet to be identified, as molecular types isolated from clinical and environmental samples have been different in western and northern Australia (12, 41, 42) and clinical cryptococcosis caused by *C. gattii* has been reported from many regions where an environmental source cannot be discerned, including parts of Australia, Africa and Papua New Guinea (12, 41, 43-45).

The regional distribution of human disease caused by *C. gattii* corresponds largely with the distribution of environmental isolates (6, 29, 34). In an exhaustive study of worldwide human clinical cryptococcosis, isolates of *C. gattii* were not found in Austria, Belgium, Denmark, France, Germany, Holland, Italy, Switzerland, and Japan but were identified at an unusually high

prevalence in Australia, Brazil, Cambodia, Hawaii, southern California, Mexico, Paraguay, Thailand, Vietnam, Nepal and Central Africa (32). Follow up studies have confirmed the high prevalence of *C. gattii* in tropical and sub-tropical regions including Brazil (27, 28), Thailand (31), Papua New Guinea (43), Venezuela (46), South Africa and Mexico (46). Small numbers of human *C. gattii* cases have been reported from India, China, Taiwan, Peru, Argentina, Rwanda, Italy (6, 22, 30, 47, 48). Cases reported from Europe and non-endemic areas of North America are thought to have been acquired elsewhere (6, 32, 49).

In North America *C. gattii* is considered to be a rarity (32). The majority of isolates have been serotype B (50, 51) with the exception of southern California where serotype C is more prevalent (32) and has been isolated from the environment (37). In Canada, cryptococcosis has been reported from most provinces, is classically associated with immunosuppression and caused by *C. neoformans* (52). *Cryptococcus gattii* has been isolated once from an AIDS patient in Quebec suffering with cryptococcosis (53) however this patient had a travel history to a region where *C. gattii* is considered endemic (37).

1.2.2 Cryptococcosis

Cryptococcosis affects humans and animals worldwide and can be caused by *C. neoformans* or *C. gattii*. While the exact mode of infection is unknown, it is widely accepted to be through inhalation of air-borne organism (23, 54). *Cryptococcus neoformans* has been isolated from the nasal passages of dogs, cats (55) and koalas (56) in Australia without evidence of infection suggesting asymptomatic colonization of the nasal mucosa following environmental exposure. It is not clear what triggers tissue invasion after colonization (6). Direct inoculation has been reported in humans and experimentally in animals (57, 58). Zoonotic transmission was proposed

in one instance in which the same molecular strain was isolated from both human and bird; however, this may represent shared environmental exposure (59). The nature of the infectious propagule is hypothesized to be the basidiospore or desiccated yeast cells. Upon entry into tissue the desiccated cell becomes rehydrated and acquires a thick polysaccharide capsule, basidiospores convert to encapsulated blastoconidia (23).

Clinical disease is dictated by host characteristics and the variety of infecting organism. *C. neoformans* is isolated most commonly from immunosuppressed individuals (29, 60). In contrast *C. gattii* is a primary pathogen as it tends to infect immunocompetent hosts. Even in areas where the organism is endemic, *C. gattii* is rarely isolated as the cause of cryptococcosis in AIDS patients (29, 60, 61).

Exposure to environmental sources of the organism is hypothesized to be the primary risk factor for clinical disease. In Australia, the aboriginal populations living in rural and semi-rural areas have a high incidence of cryptococcosis caused by *C. gattii* and further investigation suggests that this is due to a close association with eucalyptus trees (29). Men in Australia and Papua New Guinea were at an increased risk of infection with *C. gattii*, presumably because of increased contact with the organism in the environment (29, 62). Isolation of *C. neoformans* var. *grubii* from a home was a significant risk factor for AIDS patients developing cryptococcosis (21).

1.2.2.1 Animal Cryptococcosis

Clinical cryptococcosis has been reported in many domestic and wild animal species worldwide. The variety of infecting organism is often not identified due to financial constraints or the

assumption that isolates are geographically restricted and that the agent can be assumed. There are, however, differences in the clinical presentation of the different varieties that are important to recognize. These differences facilitated the identification of *C. gattii* in British Columbia.

Cryptococcosis is the most common systemic fungal infection in cats (54, 63, 64). Disease is most frequently reported in middle aged cats, but the age range is broad (65). Males have been reported to be affected more commonly than females, with the suggestion that males are more likely to be exposed for behavioral reasons (66-68). Other studies found no sex predisposition (65). Siamese cats appeared to be over represented in one Australian study (67). Contrary to the hypothesis that environmental exposure may be a principal risk factor, the disease is frequently reported in 'indoor only' cats (65, 66).

Some seasonality in feline clinical cases has been reported. In Australia there was an observed tendency for cats to present to veterinarians in the summer (67). In one study in the USA, cats presenting to the clinic with cryptococcosis were more likely to be outdoor cats in the warm seasons but strictly indoor cats in the cold seasons (66).

The three clinical syndromes most commonly reported for feline cryptococcosis include upper respiratory tract disease, dermatomycosis, and meningitis (67-69). Of the three, upper respiratory tract infection, specifically nasal cavity disease, is most commonly observed.

Clinical signs may include nasal or facial deformity, sneezing, nasal discharge, respiratory noise or coughing (65, 67, 69). Respiratory signs are often accompanied by mandibular lymph node involvement (67). Lower respiratory infection is not a common presentation in the cat.

Infection may remain localized to the nasal cavity and sinuses or penetrate through the cribriform plate to the central nervous system where it can cause meningitis. Clinical signs of feline cryptococcal meningitis include depression, ataxia, paresis, coma, lumbar pain, behavior changes, vestibular signs and seizures (65, 66, 70). Central nervous symptoms in cats are often not a primary presenting sign but secondary to respiratory infection (67). Other non-specific signs including weight loss, anorexia and lethargy are also reported in the cat (65, 66, 69).

Infection from the nasal passages may disseminate hematogenously, often presenting as cutaneous lumps or ocular lesions. Cutaneous and subcutaneous disease may involve single or multiple nodules anywhere on the body and has been reported both as dissemination and as a primary lesion (65-68, 71). Ocular lesions commonly include chorioretinitis from hematogenous dissemination or optic nerve meningitis causing blindness (63).

The role of immunosuppression in feline cryptococcosis has been debated. In an Australian case series of 29 cats, the prevalence of FIV in cases was equivalent to that in the hospital population, however, animals with both infections appeared to have more severe disease (67). In a study in the USA, 21% of cryptococcosis cases had concurrent FIV or FeLV infection, compared with 1.4% in the general hospital population. Immunosuppressed cats were more seriously affected (66). Response to therapy has been less successful in immunosuppressed cats in both Australia and the USA (67, 68). Another American case series found cryptococcosis cases with concurrent FeLV or FIV infection to have a less successful treatment outcome (65). An examination of FIV positive and negative cats in the USA found *C. neoformans* more

commonly in the oropharynx of FIV seropositive cats although no cats had signs of clinical disease (72).

Canine cryptococcosis is reported to affect relatively young dogs (73, 74). Doberman pincers, Great Danes and other large breed have been over represented relative to respective hospital populations suggesting a potential genetic or behavioral factor involved with infection (73, 75). Unlike cats and humans, there is no apparent sex predilection (73). In one retrospective study of 20 canine cryptococcosis cases in Australia, all dogs infected with *C. gattii* resided in rural or suburban environments suggesting that environmental exposure is an important risk factor (73).

The most common presentations of cryptococcosis in dogs are central nervous system, upper respiratory, ocular and cutaneous (76). Many dogs present with meningitis and clinically have ataxia, seizures, vestibular disease, cervical pain or tetraparesis (54, 76). Uncommonly cryptococcosis may present as a spinal cord lesion (77). Nasal cavity infection is less common in dogs than in cats, but may present as nasal discharge, stridor, and facial deformity (69, 73, 76). It has been hypothesized that nasal cavity involvement is more prevalent than commonly reported but that the disease goes undiagnosed in dogs until the central nervous system is involved or because the infection disseminates from the respiratory tract more rapidly in dogs than in cats (73). Optic neuritis is the most common cause of blindness in canine cryptococcosis cases but chorioretinitis is also often reported (76). Atypical presentations of canine cryptococcosis include pyelonephritis (78) and intra-abdominal masses (79).

Cryptococcosis has been reported in many other domestic and wild species. Goats with upper and lower respiratory tract disease have been reported from Australia and Spain (80-82). Guinea pigs have been infected naturally and experimentally resulting in skin and respiratory tract lesions (83-85). Ferrets have been reported to have respiratory, gastrointestinal and dermal lesions (86-88). Llamas with cryptococcal meningitis and alpacas with pneumonia are not uncommon (89, 90). Horses have been reported with cryptococcal pneumonia, rhinitis, meningitis, sinusitis and abdominal cryptococcal granulomas (91-96). *Cryptococcus* has been isolated from the reproductive tract and known to cause abortion in mares (97-99). Clinical disease has been observed in sheep (12). Mastitis has been reported in goats and cattle (100).

Avian cryptococcosis has been reported worldwide in many species (24, 101-105). Clinical conditions may vary depending on geography and infecting variety; Australian parrots were commonly infected with *C. gattii* as a primary pathogen. Cases reported from Europe and North America had more severe disease and extensive dissemination from the lung to other systems (24).

Much research has gone into cryptococcosis in the koala (*Phascolarctos cinereus*) as their association with eucalyptus trees in Australia makes them a species with a high probability of exposure (106). Koalas have been identified to have both clinical disease and sub-clinical infections (56, 106-108). There are numerous reports of cryptococcosis in non-human primates including a squirrel monkey (*Saimiri sciureus*), a common marmoset (*Callithrix jacchus*), tree shrews (*Macroscelides proboscides*, *Tupaia tana* and *Tupaia minor*), patas monkey (*Erythrocebus patas*) and rhesus monkey (*Macaca mulatta*) (109-112). Isolation of

Cryptococcus species have also been made from a wild fox (*Vulpes vulpes*) (113), a striped dolphin (*Stenella coeruleoalba*) (114), a bottlenose dolphin (*Tursiops truncatus*) (115) a cheetah (*Acinonyx jubatus*) (116) and an eastern water skink (*Eulamprus quoyii*) (117).

Cryptococcosis in animals is routinely diagnosed on the basis of histology, cytology and serology; fungal culture is less common. As a result it is often difficult to determine the exact geographical distribution of cryptococcal varieties in animal populations. One study has suggested that culture may be less useful in veterinary medicine as *C. gattii* is commonly thought to be restricted to tropical and sub-tropical regions (63). Where the variety of infecting organism has been determined in animals the pattern appears to follow that of environmental isolates and clinical human cases (73, 81).

1.3. Conclusion

Cryptococcosis is a sporadic disease of humans and animals with a global distribution. The variety of infecting organism has historically been restricted by geography and the pattern of clinical isolates follows that of environmental discovery. The recent isolation of *C. gattii* serotype B from humans, animals and the environment of southwestern British Columbia, Canada (118) challenges the previously accepted ecology of this organism and dictates the need for investigation into the emergence of the organism in this new environment.

1.4. Thesis objectives

The objectives of this study were to document the pattern of clinical *C. gattii* infection in humans and animals of British Columbia from 1999-2003, to describe the clinical presentation, outcomes and variables influencing survival of canine and feline *C. gattii* infections, to identify risk factors

for clinical *C. gattii* infection in dogs and cats residing on Vancouver Island, to identify the prevalence, and outcomes, of sub-clinical cryptococcosis and asymptomatic carriage of *C. gattii* in the nasal passages of dogs and cats and to identify terrestrial mammalian wildlife species and horses that have been exposed to or infected with *C. gattii* on Vancouver Island.

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2. The emergence of *Cryptococcus gattii* in British Columbia, Canada: 1999-2003

2.1. Introduction

In 2001, an increased incidence of cryptococcosis was identified on southern Vancouver Island, British Columbia (BC), Canada. All preliminary animal and human isolates available for culture from BC were *C. gattii* serotype B; clinical disease was recognized in humans, dogs, cats, ferrets, porpoises, and llamas resulting in the first multi-species outbreak of cryptococcosis (1). The following chapter documents the descriptive epidemiology of this outbreak of *C. gattii* in humans and animals as it emerged as an important pathogen in the temperate climate of BC between 1999 and 2003.

2.2. Methods

2.2.1. Human Cases

Human cases diagnosed between January 1999 and December 2003 were identified by the BC Centre for Disease control both retrospectively, through the Public Health Information System (PHIS), and prospectively, through reporting of microbiologists and physicians. The human case definition was specific for *C. gattii* and required clinical symptoms of cryptococcosis and isolation of *C. gattii* from a normally sterile site, or HIV negative status with clinical evidence of cryptococcosis and one of: isolation of *Cryptococcus* spp. of unknown variety from a normally sterile site, cryptococcal organism visualized in cerebral spinal fluid (CSF), cryptococcal antigen titer >1:8 in the CSF or histological identification of the organism. Probable cases had clinical

symptoms and isolation of *C. gattii* from sputum with no other causal organism present. Case data collected included location of residence, date of laboratory diagnoses, and microbiological findings.

2.2.2. Animal Cases

Animal cases diagnosed between January 1999 and December 2003 were identified prospectively and retrospectively through local veterinarians, record reviews and case reporting from private and public veterinary diagnostic labs. A confirmed case of animal cryptococcosis due to *C. gattii* required clinically compatible illness and culture of *C. gattii* from a normally sterile site. A probable animal case included any animal residing on or with a travel history to Vancouver Island in the previous two years with clinically compatible illness and a laboratory confirmed diagnosis of cryptococcosis by one of: cytology, histopathology, serum or CSF cryptococcal antigen titer $\geq 1:2$. Upon receipt of owner and veterinary consent, medical records or case summaries were obtained. Data collected included geographic location of primary residence, date of diagnosis and microbiological findings.

2.2.3. Microbiology

Culture material from clinical samples were submitted to Dr. Karen Bartlett at the University of British Columbia School of Occupational and Environmental Hygiene where there were plated onto Bird Seed Agar and incubated at 30°C. Plates were checked for growth daily for ten days before being regarded as negative. Colonies conforming to cryptococcal morphology were serotyped using capsular antibodies (Crypto-check, Iatron Laboratories, Tokyo, Japan).

2.2.4. Geographical Analysis

ArcView[®] 3.2 (Environmental Systems Research Institute Inc, Redlands, CA) was used to map the average annual incidence of human, canine and feline cases per 100,000 individuals by local health areas on Vancouver Island. Canine and feline populations were estimated by converting human census data (Statistics Canada, Census of Canada, 2001. Ottawa, ON) to animal population using a factor of 0.58 dogs and 0.66 cats per household (2). Geographical analysis focused on Vancouver Island; mainland cases with and without travel histories were excluded from maps as no relevant denominator data was available.

2.3. Results

One hundred and fifty six animal (63 confirmed and 93 probable) and 91 human (51 confirmed, 38 probable, 2 of unknown classification) cases were identified and met the inclusion criteria between January 1999 and December 2003. The majority of animal cases were feline (figure 2.1) or canine but cryptococcosis was reported in Dall's and harbour porpoises (*Phocoenidae dalli*, *Phocoena phocoena*), llamas (*Lama glama*), three avian species (eclectus parrot, *Eclectus roratus*, lesser sulphur-crested cockatoo, *Cacatua sulphurea*, cockatoo of unknown species), domestic ferrets (*Mustela putorius furo*) and a horse. In five animal cases the species of animal was unknown. These animals are most likely canine or feline cases as material was submitted from small animal practices.

Date of diagnosis was obtained for 148 animal cases and 89 human cases. Figure 2.2 shows the number of confirmed and probable animal and human cases diagnosed by year. Confirmed and probable case counts by month are reported in figure 2.3.

Location of primary residence was available for 140 animals (73 feline, 51 canine, 16 other species) and all 91 human cases. The average annual incidence of cryptococcosis per 100,000 individuals by local health areas on Vancouver Island are shown in figure 2.4 for humans and figures 2.5 and 2.6 for dogs and cats respectively. Mainland cases were not mapped. Two dogs with cryptococcosis resided on the mainland but had a travel history to the island in the previous year. Two cats and one llama from the mainland had no travel history to the island.

2.4. Discussion

The identification of *C. gattii* in Canada is an important finding that challenges the previously accepted ecology and epidemiology of the organism. Retrospective analysis of human cryptococcosis in British Columbia prior to 1999 failed to reveal any cases that met the inclusion criteria for this study suggesting the emergence of disease in this region in 1999 (McDougall, unpublished). In contrast, review of animal cryptococcosis cases identified through two diagnostic labs for the province revealed four cases of cryptococcosis in animals between 1995 and 1999, three of which were from veterinary clinics on Vancouver Island. Fungal culture is not routine in veterinary medicine so it is unknown if these cases were *C. gattii*, however given the relatively low animal population on Vancouver Island relative to the remainder of the province serviced by this diagnostic laboratory these cases suggest an earlier emergence of the organism within the region.

The number of human cases increased from 1999 to 2002 but remained relatively stable in 2003. Human case counts correspond to 8.5 per million people in 1999, 26 per million in 2000, 24 per million in 2001, 35 per million in 2002 and 2003; the incidence of human cryptococcosis in BC prior to 1999 was 1-2 per million (McDougall, unpublished). In contrast, animal cases increased

consistently with a sharp jump in 2003. This sharp increase may reflect increased testing for the agent or better reporting by veterinarians. As practitioner awareness grew, more veterinarians were testing specifically for cryptococcosis which shows few abnormalities on routine hematology (3). Although record reviews were conducted at the two largest diagnostic labs in the province, cases that were diagnosed in clinic or by different laboratories may not have been recorded. The total count of animal cases likely underestimates the true incidence of disease in the area (3).

Examination of the human and animal cases by month failed to show a seasonal pattern of disease. Some seasonality in feline clinical cases has been reported in Australia where there was an observed tendency for cats to present in the summer (4) and in the USA where cats presenting with cryptococcosis were more likely to be outdoor cats in the warm seasons and in the cold seasons were strictly indoor cats (5). Seasonal trends in the incidence of human cryptococcosis (6) or other species (7) have not been reported.

Regardless of the potential underestimation of animal cases there were significantly more clinical cases in animals compared to humans within this and previously reported time periods (3). Human cases were identified through computerized health records while animal cases were sought out by contacting veterinarians and diagnostic facilities individually. While it may be argued that the animal investigation involved more personal contact with diagnosticians, the human PHIS system and database compiled by the British Columbia Center for Disease Control was exhaustive and it is highly unlikely that laboratory diagnosed human clinical cases were missed. Within animal species there appears to be some degree of species susceptibility or

variation in species exposure as the incidence of disease is reportedly greater in cats than in dogs (8). Estimates of the incidence of cryptococcal disease in animals relative to humans worldwide are largely imprecise or unavailable as there is no formal surveillance for the disease.

Therefore, it is difficult to evaluate the relative susceptibilities of species on Vancouver Island. In Australia, koalas have been successfully used to identify geographic areas with a high-grade presence of *C. gattii* in the environment (9, 10), however it is difficult to distinguish species susceptibility from increased environmental exposure.

Human and animal cases are clustered on the east coast of the island within the Coastal Douglas-fir (CDF) biogeoclimatic zone. This area encompasses a small part of southeastern Vancouver Island, some small islands in the Strait of Georgia and a narrow strip of the adjacent mainland. The CDF region is characterized by its wet, mild winters and dry, warm summers (11). Since 2001 *C. gattii* has been repeatedly and consistently isolated from soil, air and vegetation within the CDF zone (12, 13). Maps of average annual incidence of cryptococcosis for humans, dogs and cats reveals a similar pattern of cases clustered on the southeastern coast of the island. Feline cases appear to be restricted to fewer local health areas while canine and human cases were more evenly distributed. This may reflect the travel pattern of humans and dogs relative to cats. Census data is not collected for companion animals in Canada. As a result population denominators were calculated based on a survey done by the American Veterinary Medical Association (AVMA) to estimate the population of companion animals within a community. While data was provided for different regions of the United States, the national average was used for this Vancouver Island study as no single region in the United States is representative of the Vancouver Island demographic. While this statistic may under or overestimate the actual pet

population on the island, the AVMA study is the most comprehensive survey of pet populations in North America and thus the least subjective means of estimating pet populations in the region. Calculation of incidence in this way facilitates a crude comparison of incidence between regions of Vancouver Island but the extrapolation of this conversion factor should be made with caution when evaluating variables such as population risk.

By December 2003 there were at least three animals but no people diagnosed with *C. gattii* serotype B in the lower mainland area of British Columbia that lacked travel history to the affected biogeoclimatic zone on Vancouver Island. Given the potential for earlier onset of clinical disease animals and documented higher rate of disease in animals compared to humans these cases may reflect environmental organism in a larger area than previously considered. At the time of writing no environmental source of *C. gattii* has been reported on the mainland of British Columbia.

Molecular research has identified eight molecular types within pathogenic species of *Cryptococcus* spp. (14-17). Serotypes agree with molecular types in both varieties of *C. neoformans* however studies indicate that the *C. gattii* serotypes B and C do not correlate to the four identified molecular types for this variety (17). This variation emphasizes the importance of molecular typing over serotyping in epidemiology studies. Investigation into the molecular type of isolates from humans, animals and the environment will provide valuable information on the epidemiology of the organism in this region.

Spatial, temporal and microbiological data from clinical cases on Vancouver Island reflect the linked nature of the emergence of clinical disease caused by *C. gattii* serotype B within this temperate region of the world. Further molecular and epidemiological studies are needed to identify risk factors and other variables related to the emergence of this organism within a previously unexpected area.

Figure 2.1: Confirmed and probable *C. gattii* cases by animal species on Vancouver Island from January 1999 to December 2003

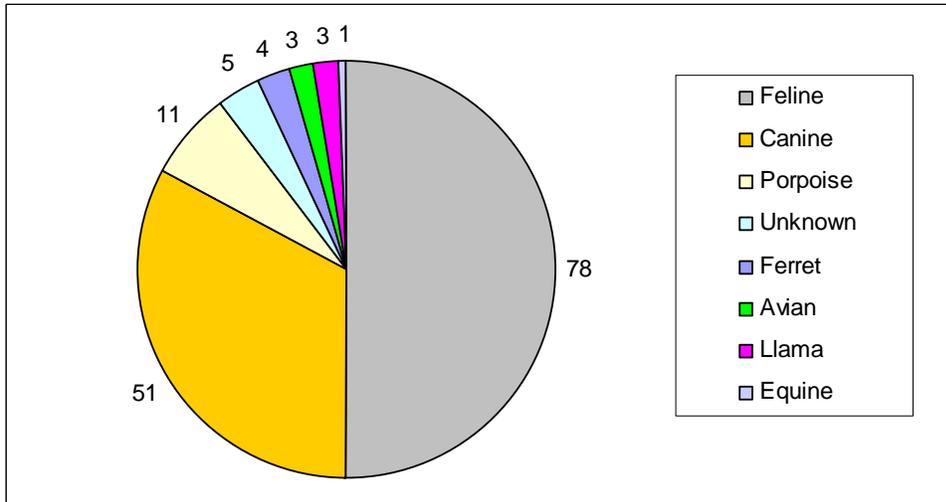


Figure 2.2: Confirmed and probable human and animal *C. gattii* cases on Vancouver Island by year from January 1999 to December 2003

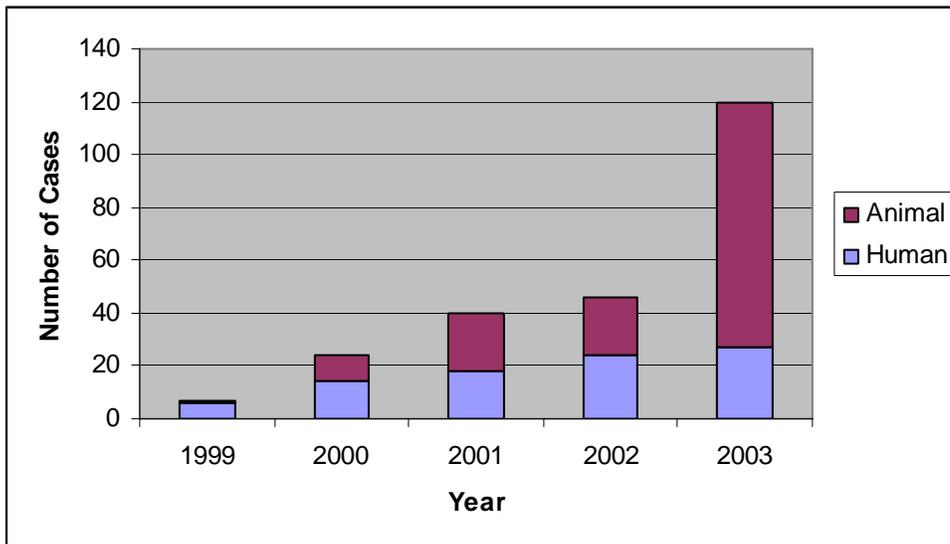


Figure 2.3: Confirmed and probable human and animal *C. gattii* cases on Vancouver Island by month from January 1999 to December 2003

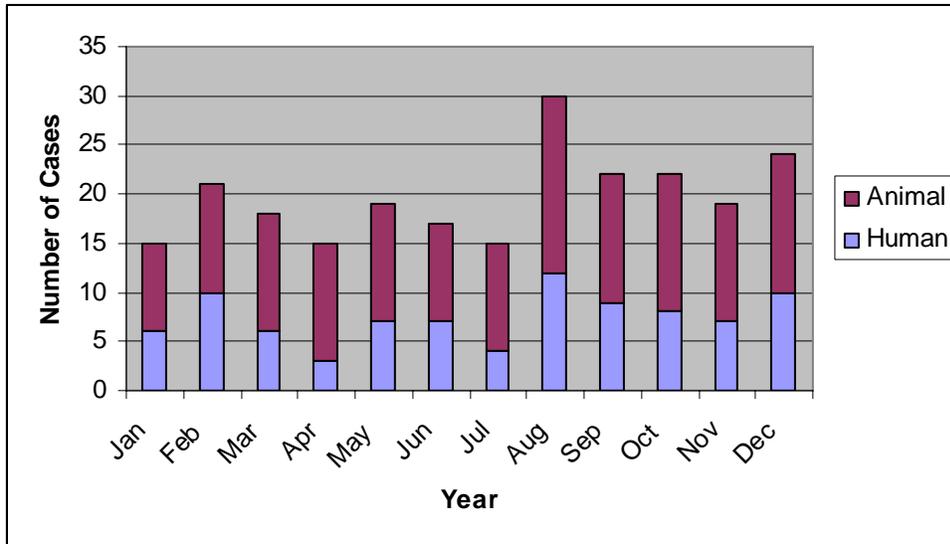


Figure 2.4: Average annual incidence of human cases per 100,000 people by local health area on Vancouver Island, BC

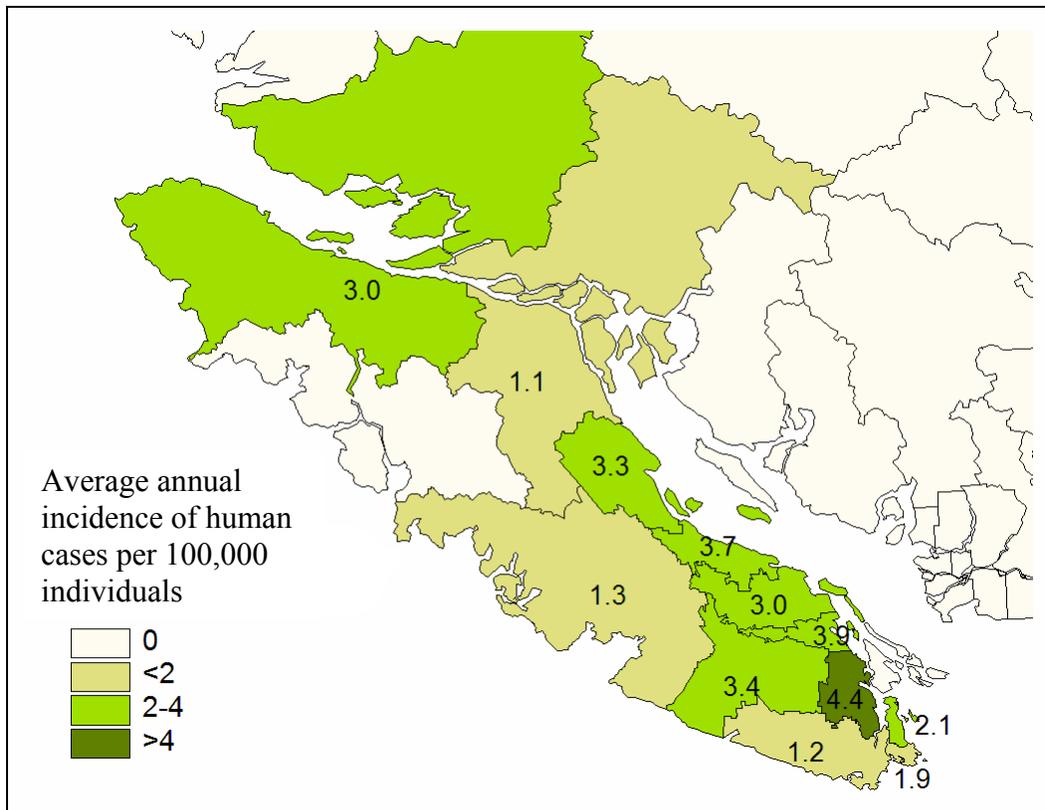


Figure 2.5: Average annual incidence of canine cases per 100,000 by local health area on Vancouver Island, BC

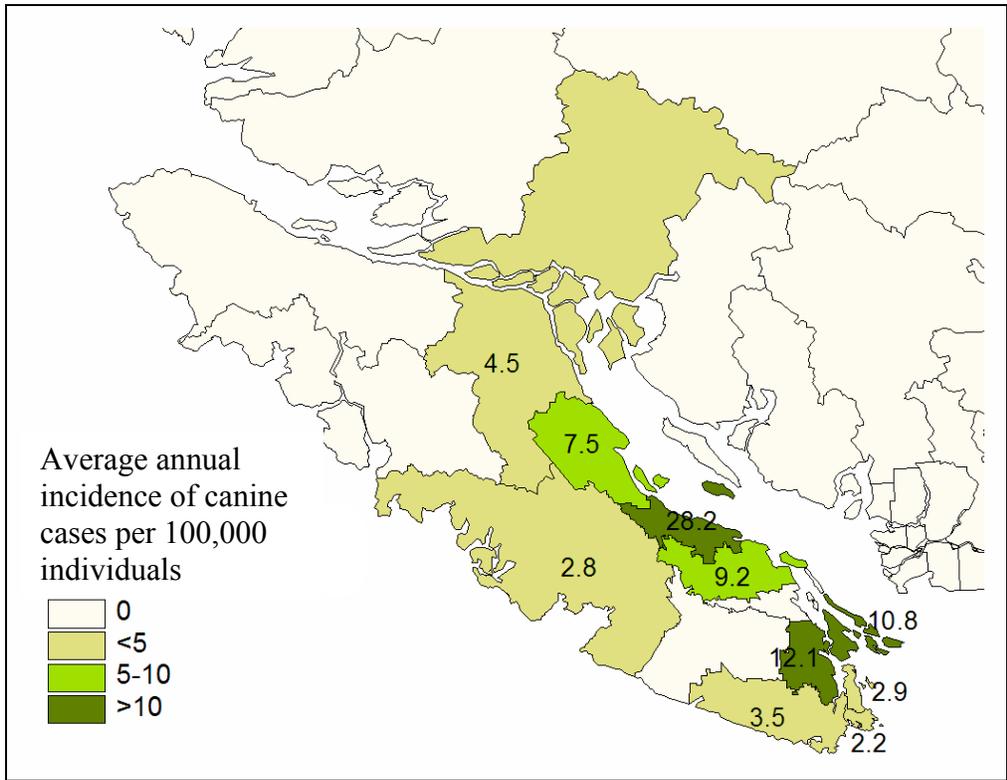
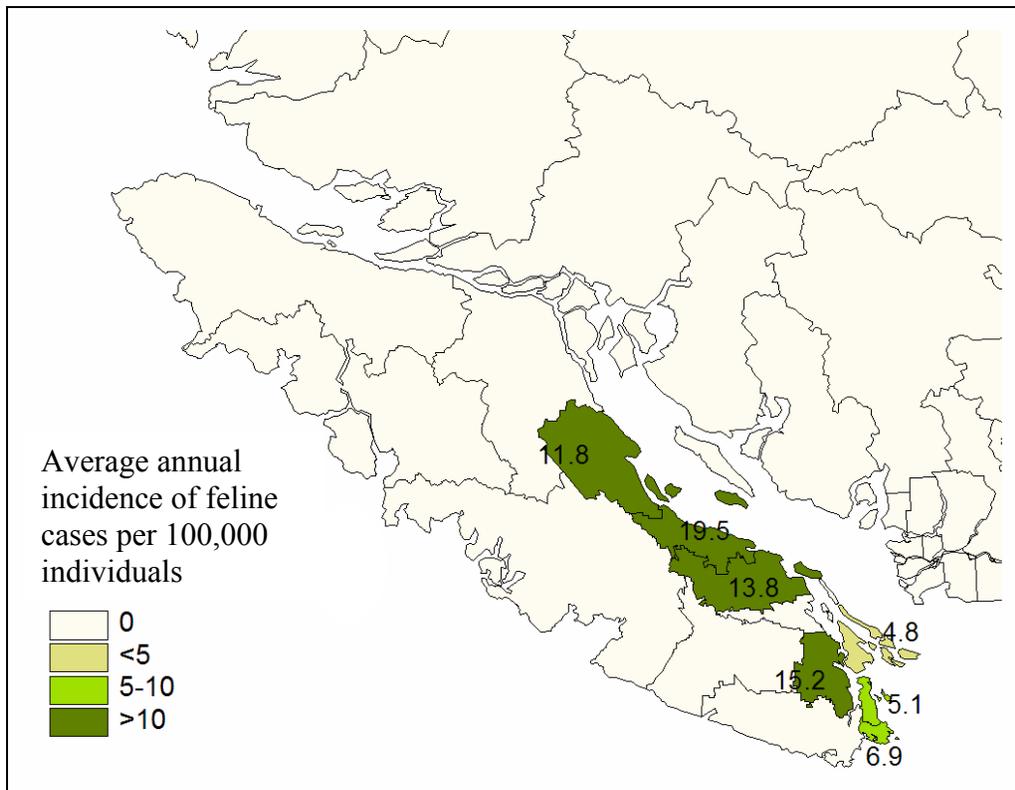


Figure 2.6: Average annual incidence of feline cases per 100,000 by local health area on Vancouver Island, BC



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3. Clinical characteristics and predictors of mortality for *Cryptococcus gattii* infection in southwestern British Columbia, Canada

3.1. Introduction

Cryptococcosis is a fungal disease found worldwide in human and animal populations. The causative agent is the organism *Cryptococcus* spp. which is considered infectious only as a desiccated yeast cell or basidiospore as found in the environment (1). The genus *Cryptococcus* includes over 37 species however only *C. neoformans* and *C. gattii* are commonly considered to be pathogenic. Conventional nomenclature included three recognized varieties of *Cryptococcus neoformans*: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *gattii* (serotypes B and C) as well as a hybrid of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (serotype AD) (2-4). Recently proposed changes to the taxonomy suggest that *C. neoformans* should be divided into two distinct species including *C. neoformans* (serotypes A, D and AD) and *C. gattii* (serotypes B and C) based on genetic variability and lack of evidence for genetic recombination between the two varieties (5).

Historically the organism responsible for clinical disease has been thought to be determined by environmental and ecological factors. *Cryptococcus gattii* had been restricted to the tropics and sub tropics while *C. neoformans* has a global distribution (1, 6). The pattern of clinical disease corresponds with the distribution of the ecologically limited environmental isolates (1, 7, 8). The epidemiology of cryptococcosis depends largely on the species of infecting organism as *C. neoformans* infects predominantly immunocompromised hosts while *C. gattii* has not been associated with a suppressed immune system (1, 9). Historically only *C. neoformans* has been

routinely isolated from animals or humans in Canada without a travel history to a region in which *C. gattii* is endemic.

In 2001 the public health authorities and veterinary community of southwestern British Columbia (BC), Canada recognized an increased incidence of human and animal cryptococcosis (10, 11). *Cryptococcus gattii* serotype B was isolated from human and animal cases associated with the outbreak and affected individuals had a history of travel to, or residence on Vancouver Island (10, 11). Investigation into the environmental niche of the fungus in Canada revealed the same organism in the environment within the Coastal Douglas Fir biogeoclimatic zone on the south east coast of Vancouver Island (12, 13). The following case series describes the clinical presentation, outcomes and variables influencing survival of canine and feline cryptococcosis cases caused by *C. gattii* in British Columbia between January 1999 and December 2003.

3.2. Materials and methods

Feline and canine cryptococcosis cases diagnosed between January 1999 and December 2003 were identified both prospectively and retrospectively from May 2003 through active surveillance and passive reporting of cases by BC veterinarians, private veterinary laboratories and the Animal Health Center at the Ministry of Agriculture, Food and Fisheries. A confirmed case of cryptococcosis due to *C. gattii* required clinically compatible illness and culture of *C. gattii* from a normally sterile site. A probable case included any animal residing on or with a travel history to Vancouver Island in the previous two years with clinically compatible symptoms and a laboratory confirmed diagnosis of cryptococcosis by one of: cytology, histopathology or a latex cryptococcal antigen agglutination test titer > 1:2 from serum or cerebral spinal fluid.

Upon receipt of owner and veterinary consent, medical records or case summaries were obtained for animals included in the study. Information collected included geographic location, animal signalment, medical history, date of presentation and diagnosis, presenting complaint and physical exam findings, diagnostic procedures and treatment. Outcomes were evaluated by contacting primary veterinarians between six months and two years after diagnosis.

3.2.1. Statistical analysis

Results of the medical record reviews were stratified by species. Based on veterinary records an animal was classified by presenting complaint and physical exam findings into a category of principal body system involved. These categories included respiratory, central nervous system (CNS), subcutaneous mass or dermal lesions, gastrointestinal, generalized illness or other. Subsequent progression of disease to additional body systems were classified as secondary and tertiary systems based on chronological order and veterinary evaluation. Descriptive and comparative statistics were computed using SPSS 12.0 (SPSS Inc., Chicago, Il., USA).

Survival analysis was conducted on all cases presenting with respiratory or CNS symptoms using SPSS 12.0 (SPSS Inc., Chicago, Il., USA). Endpoints were determined to be death due to or euthanasia because of cryptococcosis. Cases lost to follow-up or still alive at the time of follow-up were censored by the date they were last seen by the diagnosing veterinarian or last date known to be alive. A dichotomous variable, CNS symptoms, was created for animals that presented with or progressed to CNS disease. To evaluate the potential role of immunosuppression on survival, three individual variables were created; the presence or absence of significant illness within two years of diagnosis, history of steroids in the year prior to

diagnosis and the combination of steroids and disease history into a dichotomous variable representing any potential medical or pharmacological induced immunosuppression. These and other categorical variables including sex, species, antifungal therapy received, primary presenting system, and location of clinic were evaluated individually using the Kaplan-Meier survival analysis. Age, a continuous variable, was evaluated independently using Cox Regression survival analysis. Variables with $p < 0.20$ on the Log rank test were included in a multivariate Cox Regression survival analysis. Within the Cox Regression model only variables with $p < 0.05$ on the Wald test remained in the model.

3.3. Results

3.3.1. Feline

Seventy-eight feline cases suggestive of *C. gattii* infection were identified between January 1999 and December 2003. Of these, 26 were confirmed *C. gattii* serotype B on culture and 52 were probable based on diagnosis, location and travel histories. Case information and primary system involvement was obtained for 72 and 73 cases respectively. The median age at diagnosis was 7.3 years (minimum 1.2, maximum 14.7 years). There were 41 female (3 intact, 38 spayed) and 31 male (3 intact, 28 neutered) cats.

The initial presenting complaint and veterinary evaluated primary system involved is presented in tables 3.1 and 3.2 respectively. Twenty (27%) cats were presented to the veterinarian for generalized illness including weight loss, anorexia and lethargy or behavioral changes. Twenty (27%) were presented for respiratory problems including nasal discharge, increased respiratory sounds or effort, coughing and sneezing. Twenty (27%) presented for owner identified skin

lumps and 12 (17%) presented for CNS disease including ataxia and seizures. One cat (1%) presented for dental disease.

Upon veterinary examination the respiratory tract was the primary system involved in the majority of cases (40 cats, 56%). Many of the skin lumps identified by owners were enlarged submandibular lymph nodes related to upper respiratory tract infection. Nineteen cats (26%) were classified as CNS cases and 14 cats (19%) had subcutaneous lumps or dermal lesions. Eight (20%) cats presenting with clinical respiratory tract disease progressed to central nervous system disease, one cat with a sub-cutaneous mass on its dorsal thorax progressed to central nervous system disease.

Diagnosis was based on cytology, serology and histology in 42%, 31% and 28% of the cases respectively. Serology titers ranged from 1:2 to 1:20,000 and there was no pattern between titer value and organ system involvement. One cat had a negative titer but chronic nasal discharge revealed *Cryptococcus* spp. on cytologic examination.

Fifteen (21%) cats had a reported history of underlying illness that including feline leukemia virus (2), hyperthyroidism (4), feline lower urinary tract disease (3), irritable bowel syndrome, renal disease, vaccine reaction within year of diagnosis, allergies, feline asthma and chronic ectoparasite infestation. Eight cats had received steroids in the year preceding diagnosis.

Twenty-three (32%) cats were diagnosed post-mortem or euthanized upon diagnosis. Treatment was attempted in 49 (68%) cases. Of those where treatment was initiated 31 (63%) were

respiratory cases. Seven of these respiratory cases were alive at the end of the study and were still being treated with fluconazole (3), itraconazole (3), ketoconazole (1) between 5 and 10 months after diagnosis. Fourteen respiratory cases died or were euthanized within three days to eight months after diagnosis because disease had progressed to the CNS or the animal failed to respond to therapy. Seven respiratory cases were classified as clinically recovered by the diagnosing veterinarian. These animals received fluconazole (4), itraconazole (2) or fluconazole with amphotericin B. Three respiratory cases were lost to follow-up after initiation of treatment.

Eight (16%) cats receiving therapy had veterinary classified presenting symptoms consistent with central nervous system disease. Three cats were still undergoing therapy with itraconazole between five and 11 months after diagnosis. Two cats died within two weeks of diagnosis after being treated with itraconazole, another three cats were euthanized between two weeks and five months of treatment with itraconazole and fluconazole.

Ten (20%) cats with subcutaneous masses received antifungal therapy. Two of these cats were deemed recovered by the diagnosing veterinarian, both had had the masses surgically excised and one had received itraconazole for one month following excision. One cat was still undergoing treatment with fluconazole 11 months after diagnosis. Five cats were euthanized, three within one year of diagnosis, one of which had progressed to central nervous system disease within two weeks of diagnosis. The remaining two cats were lost to follow-up.

Excluding cases lost to follow-up the overall case fatality of cats included in this case series was 70%. Of those cases where treatment was initiated the case fatality rate was 55%.

3.3.2. Canine

There were 51 canine cases suggestive of *C. gattii* serotype B infection identified between January 1999 and December 2003. Nineteen were confirmed *C. gattii* and 32 cases were classified as probable. Case information was available for 50 cases. There were 23 males (7 intact, 16 neutered) and 27 females (4 intact, 23 spayed). The median age was 2.3 years (minimum 5 months, maximum 15.5 years).

The primary presenting complaint and veterinary-evaluated primary system involved is presented in tables 3.1 and 3.2 respectively. Nineteen (38%) dogs were brought to the veterinary clinic for respiratory problems. Reported complaints included nasal discharge, epistaxis, noisy breathing, sneezing and coughing. Fifteen (30%) dogs presented for neurological disease including ataxia, neck pain or seizures. Six (12%) dogs presented for generalized illness including anorexia and weight loss and six (12%) dogs presented to the veterinarian for ocular problems, four with acute onset of blindness and two with exophthalmous. Three (6%) dogs were brought in for subcutaneous lumps on the head or body. One dog presented for an acute onset of vomiting and diarrhea.

Upon examination by the veterinarian, 26 (52%) of the cases were deemed to be respiratory in nature, primarily restricted to the upper respiratory tract. Four dogs presenting with or determined to have exophthalmous on veterinary exam all had concurrent upper respiratory tract disease. Twenty-one (42%) dogs were classified as central nervous system cases and two (4%) had subcutaneous masses. The single gastrointestinal case was taken to surgery for an intussusception caused by an extraluminal cryptococcoma. Six cases (24%) initially classified by

the veterinarian as respiratory progressed to central nervous system disease. One dog with respiratory disease later developed subcutaneous masses.

Diagnosis was made primarily on cytology (44%) or serology (34%) but histology (18%) and culture (4%) were also used for diagnosis. Titers ranged from 1:2 to 1:25,000 and did not correlate with presenting complaint or primary system involved.

Six (12%) dogs had a history of underlying disease that could be considered potentially immunosuppressive. These conditions included immune mediated thrombocytopenia (2), mast cell tumor, lymphosarcoma, hypothyroid and a ruptured uterus. Ten dogs had received steroids within the year before diagnosis.

Twenty-four (48%) dogs were treated and 26 (52%) were diagnosed post-mortem or euthanized upon receipt of diagnosis. Of those undergoing therapy 15 (63%) were respiratory cases. Nine of the canine respiratory cases were still alive and receiving therapy between five and 13 months after diagnosis. Three dogs were euthanized after commencing treatment, one for diagnosis of lymphosarcoma and two for unknown causes. Two dogs were reported as recovered after four and 12 months of therapy with azole antifungals. One respiratory case was lost to follow-up.

Of the seven (29%) neurological cases where treatment was attempted, two dogs were still alive and undergoing treatment with azole antifungals alone or in combination with amphotericin B at the time of writing. Three of the dogs with primary neurological diseases died within one to

three weeks of commencing treatment with azole antifungals and or amphotericin B. Two neurological dogs were lost to follow-up.

One dog with a solitary subcutaneous mass underwent surgical excision of the mass and recovered with no antifungal therapy. The dog with the abdominal cryptococcoma was treated with fluconazole for 10 months post surgery and was clinically healthy but maintained a cryptococcal antigen titer >1:2. Excluding cases lost to follow-up the overall case fatality of dogs included in this case series was 68%. Of those cases where treatment was initiated the case fatality rate was 29%.

3.3.3. Survival analysis

Thirty nine feline and 20 canine cases representing animals presenting with respiratory or central nervous symptoms and receiving treatment were included in the survival analysis. Of these 51% of canine and 63% feline cases were censored. On initial univariate analysis only the presence of central nervous system disease ($p<0.01$), primary system involved ($p=0.06$) and species ($p=0.14$) were significant at the 20% level. Significant medical history ($p=0.52$), city of diagnosing clinic ($p=0.46$), sex ($p=0.37$), steroids in the previous year ($p=0.29$), antifungal treatment ($p=0.86$), potential immunosuppression ($p=0.83$) and age at diagnosis ($p=0.33$) were excluded from further models.

Using Cox Regression survival analysis, a model created with species, presence of CNS symptoms and primary presenting system revealed that only the presence of CNS symptoms was a significant predictor of mortality ($p<0.01$). A second Cox Regression model (figure 3.1) including only the presence of CNS symptoms found that those animals that present with or

progress to neurological disease are 4.3 times more likely to die than those that never show neurological symptoms (95% CI for mortality ratio 1.87, 9.89).

3.4. Discussion

During the study period, fifty percent more cats than dogs were identified for inclusion in the case series. This result likely represents the differing species susceptibilities to the organism. Cryptococcosis is the most common systemic mycoses of cats and, unlike other fungal diseases, clinical cryptococcosis has been reported in equal or greater frequency in cats than in dogs (14, 15). A previously reported subset of these BC cases identified more cases in dogs however this result may be due chance as the time interval was considerably smaller than that of this study (16).

As the sex ratio of the underlying population is unknown gender cannot be evaluated statistically however there does not appear to be a sex predisposition. Some studies of cats have identified a greater proportion of males affected and suggested that males are more likely to be exposed for behavioral reasons, however other studies found no sex predisposition (17-20). No apparent sex predilection has been reported in dogs (21).

As has been observed in other case series, feline cryptococcosis is more common in middle aged cats but cats of all ages may be affected. The age range in this and other case series is wide (17-19, 22). In contrast to cats, clinical disease was more common in younger dogs as has been previously documented (21, 23). Breed predisposition could not be evaluated in this case series as the underlying population of the region is not known. In other studies Doberman Pincers, Great Danes and other large breed have been over represented relative to respective hospital

populations suggesting a potential genetic or behavioral factor involved with infection (21, 24). Siamese cats appeared overrepresented in one Australian study (19).

Respiratory disease was the most common syndrome in cats (56%) followed by central nervous system symptoms (26%) and subcutaneous or dermal lesions (19%). While the high proportion of respiratory cases is similar to previous studies, the proportion of cats with CNS symptoms exceeded those previously reported (17, 19, 22) and may represent a difference in virulence of *C. gattii* compared to *C. neoformans* which has been more commonly isolated or, based on location, assumed to be the causative agent in the other case series. Central nervous system signs in cats have been reported to be secondary to respiratory infection and not a common primary presenting sign (19), however, this study had a high percentage of cases presenting for CNS disease without a reported history of respiratory symptoms.

The manifestation of cryptococcosis in dogs of British Columbia is very similar to those reported from Australia where a retrospective case series identified 55% respiratory cases, 35% central nervous system, 5% with disseminated disease and 5% with gastrointestinal symptoms (21). It has been hypothesized that nasal cavity or respiratory involvement is more prevalent than commonly reported but disease goes undiagnosed in dogs until the central nervous system is involved or the organism simply disseminates from the respiratory tract faster in dogs than in cats (21). Similarly, this study found a greater proportion of canine cases were classified as neurological at first veterinary visit when compared to feline cases. Intra-abdominal masses have been reported in dogs but may be considered an atypical appearance of cryptococcosis (25).

Only respiratory and CNS cases were included in the survival analysis as the relative number of other cases receiving treatment was too low to evaluate meaningfully. The results of the survival analysis reveal that the presence of neurological symptoms was a very strong predictor of mortality. Animals presenting with or progressing to CNS disease were over four times more likely to die than those never exhibiting neurological symptoms.

The poor survival in animals exhibiting central nervous system symptoms observed in this study may be explained by a number of factors. Most significant is likely the severity of the disease once it has entered the central nervous system (15, 17). It is also possible that because endpoints in this analysis included both death and owner elected euthanasia, treatment may have been terminated for reasons other than the animal being moribund. Such reasons could include cost of therapy or owner perceived severity of clinical symptoms and electing humane euthanasia. Finally survival may be influenced by treatment initiated. While antifungal therapy was not a significant predictor in the model it is important to note that only the azole antifungals were used with enough frequency to influence the model. For central nervous system cases effective therapy requires a drug that can penetrate the blood brain barrier such as amphotericin B, flucytosine and fluconazole (14, 18, 21, 26). Failure to select one of these agents would result in a poor response to therapy. Minimum inhibitory concentration (MIC) work done on a subset of cultures from this case series found all isolates were susceptible to amphotericin B but there was some intermediate sensitivity and resistance to fluconazole and flucytosine (16). Amphotericin B, the most affordable and potentially efficacious antifungal drug when dealing with CNS cases, was largely avoided in these cases out of concern for nephrotoxic side effects. Newly proposed

treatment regimes using amphotericin B need to be considered in attempt to improve animal outcomes (14).

Geographic location of diagnosing clinic was evaluated because of potential variation in diagnostic procedures by region and variability in environmental load and exposure but was not determined to effect survival. Species alone was not a significant predictor of survival however dogs have a higher proportion of central nervous presentations which may indirectly impact survival as clinical presentation will influence a veterinarian and owners decision to treat.

The veterinary-identified primary system of involvement did not significantly affect the survival model but should be considered clinically important. The presence of CNS symptoms at any point was a very strong predictor of mortality and this variable encompasses any cases who present with primary CNS disease. In this analysis there were few animals presenting with primary CNS disease, receiving treatment and then surviving long enough to be included in and contribute to the model which may explain why primary system alone was not significant. Similarly in a study on the outcomes of cats treated with itraconazole the location of infection did not affect outcome, however they also had a very small proportion of primary CNS cases (20).

The role of immunosuppression in animal cryptococcosis is unclear. Neither a history of potentially significant illness, corticosteroids in the previous year or a combination of the two variables significantly influenced the survival analysis for BC cases. In a study of cats in Australia, the prevalence of feline immunodeficiency virus (FIV) in cases was equivalent to that

of the hospital population, however these animals appeared to have more severe disease than those without concurrent FIV infection (19). In contrast, a retrospective study in the USA found the prevalence FIV and feline leukemia infection in cryptococcosis cases to be higher than the general hospital population and the concurrently infected individuals were more seriously affected (18). Response to therapy has been reportedly less successful in immunosuppressed cats in some studies (19, 20) but not in others (17). The causative agent in most of these studies has been identified as or assumed to be largely *C. neoformans* and not *C. gattii*. In humans *C. neoformans* predominantly infects immunocompromised hosts (9) while *C. gattii* has not been associated with a suppressed immune system (1). Species of *Cryptococcus* may dictate the role of immunosuppression in clinical animal cases. Based on the results of this study immunosuppression does not significantly affect the mortality of dogs and cats with *C. gattii* infection in BC.

This study provides a general summary of important descriptive case characteristics for canine and feline cryptococcosis due to *C. gattii* in BC. Given the recent emergence and apparent persistence of *C. gattii* in the region veterinarians need to be aware of the primary presenting symptoms suggestive of *C. gattii* infection and variables that may influence outcomes. The use of clinical information from multiple sources puts severe limitations on the type of information that can be used to summarize case characteristics. Variation in diagnostic and therapeutic techniques dictates broad generalizations of individual animal reports. Clinicopathologic features of a subset of these cases have been reported in more detail elsewhere (16) but clinical trials or case series with standardized diagnostic and treatment regimens are needed to better assist clinicians in making individual animal diagnoses and treatment decisions.

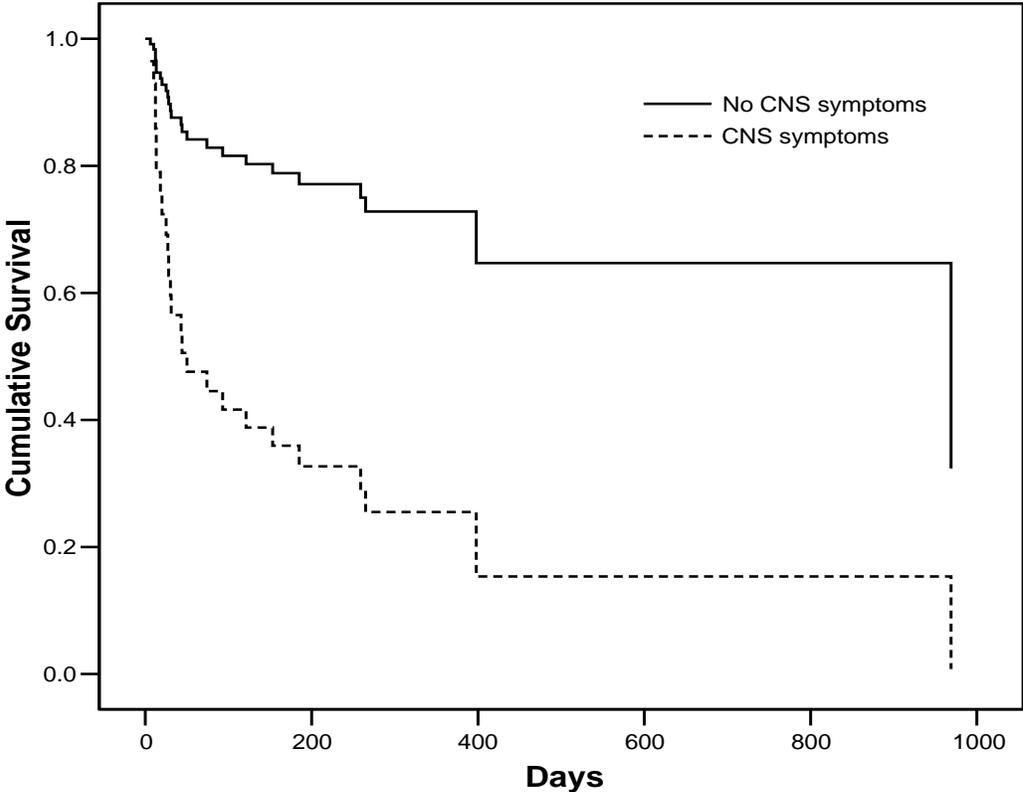
Table 3.1: Owner reported primary presenting complaint for canine and feline cryptococcosis

	Feline, n (%)	Canine, n (%)
Respiratory	20 (27)	19 (38)
Central nervous system	12 (16)	15 (30)
Generalized illness	20 (27)	6 (12)
Dermal	20 (27)	3 (6)
Ocular	0	6 (12)
Gastrointestinal	0	1 (2)
Other (dental)	1 (1)	0

Table 3.2: Veterinary reported primary organ system involved in canine and feline cases

	Feline, n (%)	Canine, n (%)
Respiratory	40 (56)	26 (52)
Central nervous system	19 (26)	21 (42)
Subcutaneous mass	14 (19)	2 (4)
Gastrointestinal	0	1 (2)

Figure 3.1: Cumulative survival for animals with and without central nervous system symptoms



3.5. References

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4. Risk factors for clinical *Cryptococcus gattii* infection in dogs and cats on Vancouver Island, British Columbia, Canada

4.1. Introduction

Cryptococcosis is a sporadic disease found worldwide in human and animal populations. The causative agent is *Cryptococcus* spp. which is considered infectious only as a desiccated yeast cell or basidiospore as found in the environment (1). The genus includes many species but only *C. neoformans* and *C. gattii* are commonly regarded as pathogenic. *Cryptococcus neoformans* has two recognized varieties: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) as well as a hybrid of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (serotype AD) (2, 3). *Cryptococcus gattii* (serotypes B and C) has recently been identified as a species distinct from *C. neoformans* based on genetic variability and lack of evidence for genetic recombination between *C. neoformans* and *C. gattii* (4).

Cryptococcus gattii had historically been geographically confined to the tropics and subtropics while *C. neoformans* had a global distribution (1, 5); the pattern of clinical disease corresponded largely with the ecologically restricted environmental organism (1, 6, 7). Since 1999 *C. gattii* (serotype B) has been isolated from sick people and animals in southwestern British Columbia (BC) as well as from air, soil and vegetation samples collected on the south east coast of the island (8-11). These findings are in stark contrast to the organisms previously described ecology and associated epidemiology.

Prior to the emergence of *C. gattii*, cryptococcosis was considered a rare disease of companion animals in Canada. Vancouver Island veterinarians were challenged by an increasing number of cases and a lack of information on risk reduction measures. The purpose of this study was to identify risk factors for clinical *C. gattii* infection in dogs and cats residing on Vancouver Island, BC.

4.2. Materials and methods

4.2.1. Inclusion criteria

As two previously reported case series of animal cryptococcosis identified a relationship between *C. gattii* infection and southeastern Vancouver Island, BC this area was selected as a focal point for the study (8, 11). Cryptococcosis in animals is not a reportable disease in Canada so incident cases of feline and canine cryptococcosis meeting the case definition were identified by contacting veterinary clinics on Vancouver Island and private diagnostic laboratories servicing BC. Cases included in this study were identified during two distinct time periods; August 2001 to February 2002 and May to December 2003.

A confirmed case of cryptococcosis due to *C. gattii* required clinically compatible illness (12) and culture of *C. gattii* from a normally sterile site. A probable case included any animal residing on or with a travel history to Vancouver Island in the previous two years with clinically compatible symptoms (12) and a laboratory confirmed diagnosis of cryptococcosis by one or more of: cytology, histopathology or a latex cryptococcal antigen agglutination test titer > 1:2 from serum or cerebral spinal fluid. Many cases were diagnosed without material for fungal culture. Given that *C. gattii* was identified in virtually all previously reported cases of animal

cryptococcosis on Vancouver Island during the study period where culture material was available, confirmed and probable cases were included in the analysis (8, 11).

Control animals were obtained from the veterinary clinic that diagnosed the case. Controls were matched on species, age (within 1 year of case age) and dogs were size matched where large dogs were defined as > 20 kg and small dogs were < 20 kg. Matching was used to control confounding by these variables and increase the power of the study as sample size was expected to be small. Veterinary clinics were sent a letter outlining the control animals required and, beginning on a random day, asked any client presenting to the veterinary clinic whose animal met the control description if they would take part in the study. If they refused, the next animal meeting the description was asked until all controls were obtained. Exclusion criteria for control animals were those meeting the match requirements but presenting to the veterinary clinic with a diagnosis of, or clinical symptoms strongly suggestive of, cryptococcosis.

4.2.2. Interview

A standardized questionnaire was administered over the telephone or by personal interview for cases and over the telephone for all controls. The questionnaire focused on two main areas; environmental variables and animal characteristics. Controls were asked to answer the questions with reference to the same time period as the matched case. The number of questions in the questionnaire was modified slightly between the two sampling periods as the investigation into *C. gattii* in the region identified other potential risk factors for infection.

Environmental variables focused on factors that may increase exposure of the animal to the organism. This included proximity to wooded areas or farmland, contact with eucalyptus trees or

products and owner activities six months prior to case diagnosis such as: hiking, gardening, chopping wood, handling birds/cleaning bird roosts or digging soil. They were also asked about activities taking place within ten kilometers (km) of the animal's primary residence in the six months preceding diagnosis that may disturb the environment such as construction, logging or commercial soil disturbance.

Host characteristics explored included gender of the animal, travel on and off of Vancouver Island in the year preceding diagnosis, activity level, presence of potentially stressful changes or owner perceived stressors in the previous year, history of disease, medication, vaccination and owner supplementation with commercial products. Animal-environment interactions including hunting and digging in soil were also included. Owners were asked how much time their animal spent outside a 10 km radius of their home in a given week, how many hours were spent outdoors on a given day and how many years the animal had lived within the municipality.

All questions were close ended. Animal activity level was recorded on a four point scale (very low, low, high, very high) reflecting how active owners felt their pet was in certain environments. Times spent outdoors, in the municipality and outside a radius of 10 km of the home were continuous variables; the remaining variables were dichotomous.

4.2.3. Statistical analysis

Odds ratios and 95% confidence intervals were calculated from McNemar chi-squared values for cases versus controls for each potential risk factor (13). Continuous variables were evaluated using the paired t-test. For animal activity level the four point response scale was converted to a dichotomous variable with very low and low activity classified as below average while high and

very high were classified as above average. Data from both species were pooled together for the initial analysis and statistically significant risk factors were stratified by species for further evaluation.

4.3. Results

Over 80 owners of clinical cases were interviewed but only 17 matched controls in 2001/2002 and 32 in 2003 were provided by the diagnosing veterinary clinic. Less than 10 cases meeting the case description were not interviewed because the owner or diagnosing veterinarian was unwilling to participate in the study. Based on available clinical information these cases did not differ from those included. In total 49 cases and matched controls were included in the analysis.

The results, pooled by species, are presented in table 1; only statistically significant risk factors and continuous variables are reported in the text. Logging and soil disruption within a 10 km radius of the primary residence were the most significant risk factors for clinical cryptococcosis with those exposed being 14 (3.00, 65.37) and 18 (4.21, 76.93) times more likely to develop disease, respectively. Animals that had traveled on Vancouver Island within the last year were 6 (1.61, 22.33) times more likely to become infected than those without a travel history in the previous 12 months. Likewise cases spent a mean of 2.2% of time outside of a 10 km radius of the primary residence while controls spent only 0.33%; a difference that was statistically significant ($p=0.027$). Owners who went hiking or visited a botanical garden recently were 4 (1.61, 9.90) and 5 (1.28, 19.60) times more likely to have animals become diseased. Activity level of the animal was a significant risk factor as animals with owner classified activity level of above average in the house and outside unrestrained were 3 (1.02, 8.80) and 8 (1.40, 45.89) times more likely to become diseased, respectively.

Owner administration of supplements increased the odds of disease 3.67 (1.11, 12.07) times. The odds ratio for owners knowing people with cryptococcosis or other people with animals diagnosed with cryptococcosis was 5.67 (1.91, 16.79). Medication in the previous year and the presence of other pets were both considered protective in the crude analysis. The amount of time an animal spent outdoors ($p=0.498$) or living in their current home municipality ($p=0.703$) were not significant.

Results of stratification of the statistically significant risk factors by species are presented in table 4.2. Logging and soil disturbance remained the greatest risk factors for disease. Odds of disease in the presence of logging was increased 17 (2.12, 136.40) times in dogs and 6 (0.94, 38.48) times in cats; however, only dogs remained statistically significant. The measure of effect of soil disturbance was 17 (2.12, 136.40) and 20 (1.90, 52.76) for dogs and cats, respectively, and remained statistically significant for both species. The odds of disease in dogs, but not in cats, was statistically increased when the animal had traveled on Vancouver Island in the previous 12 months. Canine cases spent significantly more time outside a 10 km radius of their home compared to controls ($p=0.024$) but the difference was not statistically significant for feline cases ($p=0.33$).

The odds of disease by species was similar to the pooled result for owners that hiked or visited a botanical garden in the previous six months, however only hiking of cat owners remained statistically significant. When the variables reflecting animal activity level were stratified by species confidence intervals became large and none of the variables remained significant.

Supplements and knowing other cryptococcosis cases remained statistically significant risk factors for cats but not for dogs in the stratified analysis. Medication in the previous 12 months remained significantly protective for dogs but not for cats, while the presence of other pets was not statistically significant for either species.

4.4. Discussion

Cryptococcus is a genus of environmental fungi that can be isolated from vegetative matter worldwide. While the exact mode of infection is unknown it is widely accepted to be through inhalation of air-borne organism (14, 15). *Cryptococcus* spp. have been isolated from the nasal passages of dogs, cats (16, 17) and koalas (18) in Australia and Canada without evidence of disease suggesting asymptomatic colonization of the nasal mucosa following environmental exposure. It is not clear what triggers tissue invasion after colonization (1).

Association with environmental sources of organism has been recognized as a primary risk factor for clinical cryptococcal disease in humans. In Australia, the aboriginal population living in rural and semi-rural areas were over 12 times more likely to develop of *C. gattii* infection relative to the reference population (7). This increased risk was proposed to be due to the populations close association with eucalyptus trees, a regional environmental reservoir. Men in Australia and Papua New Guinea are also at an increased risk of infection with *C. gattii*, presumably because of increased contact with environmental organism (7, 19).

Likewise in animals, environmental exposure to *Cryptococcus* spp. is an important risk factor. Male cats have been reported infected more often than females, with the suggestion that males are more likely to be exposed for behavioral reasons; however, other studies found no sex

predisposition (20-23). In a retrospective study of 20 canine cryptococcosis cases in Australia, all dogs infected with *C. gattii* resided in rural or suburban environments suggesting increased proximity to the organism was a risk factor (24). Doberman Pincers, Great Danes and other young, large breed dogs have been over represented relative to respective hospital populations for cryptococcosis cases suggesting a potential behavioral or genetic factor involved with infection (24, 25). Unlike cats and humans, there is no evidence of sex predilection for cryptococcal infection in dogs (24).

In this study, residing within 10 km of a site of commercial soil disruption or vegetation clearing were the most significant risk factors for clinical cryptococcosis due to *C. gattii*. Physical disruption of the environment can result in more airborne particulate matter, including *C. gattii* (K. Bartlett, unpublished). In contrast, owner induced vegetation and soil disruption at the residence, such as chopping wood, bringing wood into the house, digging soil, gardening or the exposure of animals to garden related products and home construction, were not identified as significant risk factors. This suggests such activities may not result in sufficient aerosolization of particulate matter to increase the risk of infection when compared to larger scale environmental disturbance associated with logging and commercial soil excavation. Such findings imply a threshold level of air contamination exists that influences the risk of animal infection; or that the source of the infection is not in the immediate residential environment.

Animals that had traveled on Vancouver Island within the last year were six times more likely to become ill than those with no travel history. Clinical animals also spent a statistically greater percentage of time outside a 10 km radius of their primary residence than controls suggesting

they traveled more often. Stratification of these variables revealed that this pooled result was influenced more by dogs than cats as dogs are more likely to travel with their owners. Animals that travel may be at greater risk of disease because of increased opportunities for exposure. Environmental *C. gattii* has not been isolated ubiquitously on Vancouver Island (9) so travel would increase the probability of exposure to a positive site. Duration of time spent in the residential municipality and time spent outdoors daily were not significant risk factors. Similarly residing within two km of a wooded or agricultural area was not a risk factor. This supports the hypothesis that some animals are not being exposed at their home; rather in places to which they travel where there is a higher environmental load. Animals traveling off of Vancouver Island were not at increased risk of disease; however, *C. gattii* had not been isolated from the environment off of the island at the time of this study (26).

Higher activity levels in the house and outdoors unrestrained were significant risk factors for disease; the odds of disease was also very high for animals outside and restrained; however, an extremely large confidence interval deemed the measure to not be significant statistically. Behavioral factors, including activity level, may significantly increase an animal's risk of infection by bringing the individual in contact with *C. gattii* more frequently. An active animal may also disturb the environment more and aerosolize additional organisms that can be inhaled. Gender, previously suggested to be a risk factor for cryptococcosis because of behavioral reasons was not determined to be significant in this case-control study.

There was an increased risk of disease in animals whose owners hiked and visited a botanical garden in the six months preceding diagnosis. As the environmental organism is small enough to

be transported on fomites it is possible that humans who participate in outdoor recreational activities transport the organism back to their residence and unwittingly expose their pet. Dogs often accompany their owners on hiking trips and thus are at an increased risk of direct exposure through this recreational activity. Stratification of hiking by species resulted in a wide and non-significant confidence interval for dogs. This is likely the result of a small sample size.

Questions regarding visitation to botanical gardens were included to explore the role of imported vegetation, most specifically eucalypt trees, on disease. None of the direct eucalyptus variables were significant and the risk of an owner visiting a botanical garden in the period prior to diagnosis may indirectly imply owner travel or affinity for gardening and naturalist activities.

Owners who knew people with cryptococcosis or other people who had animals with cryptococcosis were over five times more likely to have a diseased pet themselves. This result may be a result of recall bias as owners with a sick animal may be more likely to recall or seek out others with the same problem. Alternatively the finding may reflect the clustering of positive environmental sites resulting in clustering of cases at the level of the neighborhood. Owner administered supplements were a significant risk factor, but protopathic bias may have influenced this finding as the incubation period is long and, in the period preceding diagnosis, owners may have attempted to supplement an animal that appeared slightly unwell. Likewise individuals who choose to supplement a sick pet may be more apt to bring the animal to the veterinarian in the case of illness or pay for diagnostic procedures that would facilitate identification of the case and inclusion in the study.

Medication in the year preceding diagnosis was identified to be protective; however this finding may have been influenced by Berkson's bias, the selection of the control animals from a hospital population. Control animals were those presenting to the veterinary clinic on a random day without a diagnosis of or clinical symptoms strongly suggestive of cryptococcosis. Because the clinic was used as the control selection point animals were more likely than the general population to be presenting to the veterinarian because of illness and therefore be receiving medication. Control selection could also have influenced the finding that owning multiple pets was considered protective when dogs and cats were pooled together. Owners of multiple pets tend to visit the veterinary clinic more often and thus have greater odds of being selected as a control.

Variables indicating potential immunosuppression including any history of disease, administration of steroids in the previous year, environmental changes or owner perceived stressors were not significantly associated with cryptococcosis cases. The role of immunosuppression in feline cryptococcosis has been debated in the literature. The prevalence of FIV in cats with cryptococcosis has been reportedly equivalent to that of a hospital population in Australia (22) while in the USA concurrent FIV or FeLV infection in cats with cryptococcosis was much higher than the general hospital population (21). An examination of FIV positive and negative cats in the USA found *C. neoformans* more commonly in the oropharynx of FIV seropositive cats, although no cats had signs of clinical disease (27). It is important to note that in many of these studies the causative agent has been identified as, or assumed to be *C. neoformans* and not *C. gattii*. Clinical disease is dictated by host characteristics and the variety of infecting organism; *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are isolated

most commonly from immunosuppressed individuals (7, 28). In contrast *C. gattii* should be considered a primary pathogen as it tends to infect immunocompetent hosts; even in areas where the organism is endemic *C. gattii* is rarely the cause of cryptococcosis in AIDS patients (7, 28, 29). This study lends further support that immunosuppression is not required for *C. gattii* infection.

The first environmental isolation of *C. gattii* was from eucalyptus trees (*Eucalyptus camaldulensis* and *E. tereticornis*) in Australia (6, 30). Subsequent to this discovery *C. gattii* has been recovered from material associated with eucalypt species in many other parts of the world including California, India and Brazil (31-34) along with non-eucalypt tree species from tropical and subtropical areas worldwide (31, 35-37). There are studies that suggest alternative environmental sources of *C. gattii* have yet to be identified, as molecular types isolated from clinical and environmental samples have been different in western and northern Australia (38-40) and clinical cryptococcosis caused by *C. gattii* has been reported from many regions where an environmental source has not been identified including parts of Australia, Africa and Papua New Guinea (38, 40-43). In this study, animal association with eucalyptus trees or products was not a significant risk factor for cryptococcosis. *Cryptococcus gattii* has been isolated from multiple tree species, soil samples and air samples on Vancouver Island, but never in association with a eucalypt tree (9). This information suggests the existence of an alternative environmental niche for the organism on Vancouver Island.

Cryptococcus neoformans has historically thought to be associated with avian excreta, particularly pigeons (32, 44-49). Pet owners handling birds or cleaning up bird roosts was not

identified as a risk factor in this case-control study. Given that avian excreta is rich in creatinine and other chemical constituents that promote fungal replication, it is likely that the environmental niche of the fungus is soil or vegetation but that the organism is easily isolated from avian excreta as it provides a good media for growth (48, 50).

Just over half of the requests for clinic matched controls were obliged. The corresponding low sample size resulted in wide confidence intervals and results that, while biologically plausible, were occasionally not statistically significant. Further stratification of potential risk factors to look for correlation between variables was not possible given the sample size restrictions. Regardless of this imposed limitation this study identified a dramatic increase in the odds of *C. gattii* in dogs and cats residing close to sites of major environmental disturbance, animals that travel on Vancouver Island and animals that are of above average activity level.

It can be concluded that where an environmental organism is not uniformly and ubiquitously distributed in the environment, risk is increased if the organism is re-distributed through disruption of its environmental niche or the likelihood of encountering the environmental cluster is increased through travel or activity level. Owners and veterinarians residing within an endemic area of environmental organism, such as *C. gattii*, should be aware of these risk factors such that risk can be mitigated or complete patient history data can facilitate prompt diagnosis. It is critical however that veterinarians discuss these risks in context as cryptococcosis remains a relatively rare disease of companion animals and the benefits of fresh air far exceed the risk of disease.

Table 4.1: Odds ratios and 95% confidence intervals for environmental and host variables

Environmental variables	n	OR	95% CI
Soil disturbance within 10 km of residence in previous 6 months	32	18	4.21, 76.93
Logging with 10 km of residence in previous 6 months	32	14	3.00, 65.37
Know other crypto case	32	5.67	1.91, 16.79
Owners visiting a botanical garden in previous six months	32	5	1.28, 19.60
Owners hiking in previous six months	32	4	1.61, 9.90
Wooded area within 2 km of residence	49	2.67	0.75, 9.55
Construction at residence	49	2.2	0.79, 6.16
Contact with eucalypt trees	49	1.67	0.40, 6.87
Animal contact with topsoil	47	1.5	0.62, 3.65
Animal contact with compost	48	1.44	0.62, 3.36
Animal contact with bark mulch	47	1.3	0.57, 2.96
Animal contact with fertilizer	47	1.25	0.49, 3.16
Farm within 2 km of residence	49	1.14	0.42, 3.15
Wood products brought into residence	49	0.92	0.42, 2.02
Owner chopping wood at residence	49	0.91	0.39, 2.14
Contact with eucalypt cuttings	49	0.91	0.39, 2.14
Contact with eucalypt products	49	0.86	0.29, 2.55
Owner digging soil	49	0.71	0.32, 1.60
Owners gardening at residence	49	0.6	0.15, 2.47
Owner contact with birds	49	0.4	0.13, 1.23
Host characteristics	n	OR	95% CI
Activity outdoors (restrained)	11	10	0.95, 105.07
Activity outdoors (unrestrained)	23	8	1.40, 45.89
Travel on Vancouver Island in previous year	49	6	1.61, 22.33
Travel off Vancouver Island in previous year	49	4	1.00, 16.75
Owner administered supplements	32	3.67	1.11, 12.07
Hunting	44	3	1.15, 7.86
Activity indoors	28	3	1.02, 8.80
Other stressors in previous year	32	2.67	0.75, 9.55
Gender	49	1.56	0.68, 3.57
Steroids in previous year	48	1.5	0.43, 5.27
Digging in soil	47	1.18	0.53, 2.64
Environmental changes in previous year	32	1	0.38, 2.66
Vaccination in previous year	49	0.73	0.29, 1.80
Any history of disease	49	0.6	0.26, 1.37
Other pets in the household	49	0.36	0.13, 0.95
Prescription medication in previous year	32	0.17	0.06, 0.49

Table 4.2: Odds ratios and 95% confidence intervals for environmental and host variables stratified by species

	Canine			Feline			Pooled		
	n	OR	95% CI	n	OR	95% CI	n	OR	95% CI
Soil disturbance within 10 km	12	17	2.12, 136.40	20	20	1.90, 52.76	32	18	4.21, 76.93
Logging within 10 km	12	17	2.12, 136.40	20	6	0.94, 38.48	32	14	3.00, 65.37
Activity outdoors (unrestrained)	12	9	0.81, 99.95	11	4	0.53, 30.31	23	8	1.40, 45.89
Animal travel on Vancouver Island	20	4.5	1.11, 18.19	29	7	0.55, 88.99	49	6	1.61, 22.33
Know other crypto case	12	9	0.81, 99.95	20	4.33	1.37, 13.68	32	5.67	1.91, 16.79
Owners visiting a botanical garden	12	5	0.33, 76.81	20	4	0.96, 16.75	32	5	1.28, 19.60
Owners hiking in previous six months	12	4	0.96, 16.75	20	4	1.24, 12.88	32	4	1.61, 9.90
Owner administered supplements	12	2	0.38, 10.56	20	7	1.16, 42.26	32	3.67	1.11, 12.07
Hunting	17	5	0.33, 76.81	27	2.6	0.96, 7.02	44	3	1.15, 7.86
Activity indoors	8	2	0.38, 10.56	20	4	0.96, 16.75	28	3	1.02, 8.80
Other pets in the household	20	0.33	0.07, 1.53	29	0.38	0.11, 1.34	49	0.36	0.13, 0.95
Prescription medication in previous year	12	0.05	0.01, 0.37	20	0.38	0.11, 1.34	32	0.17	0.06, 0.49

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5. Sub-clinical infection and asymptomatic carriage of *Cryptococcus gattii* in dogs and cats during an outbreak of cryptococcosis.

5.1. Introduction

Cryptococcosis is a fungal disease found worldwide in human and animal populations. The epidemiology of clinical disease depends largely on the species of infecting organism.

Cryptococcus neoformans var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) are globally distributed and infect predominantly immunocompromised hosts (1).

Cryptococcus gattii (serotypes B and C) has recently been recognized as a species distinct from *C. neoformans* based on molecular and mating type characteristics (2). Clinical disease caused by *C. gattii* has not been associated with a suppressed immune system (3) and has historically been restricted to the tropics and sub-tropics, particularly in association with eucalyptus trees (4-6). The organism is not contagious and considered infectious only as a desiccated yeast cell or basidiospore as found in the environment (3). Previously only *C. neoformans* has been routinely isolated from human or animal cases of cryptococcosis in Canada without a travel history to a region in which *C. gattii* is endemic.

In 2001 an increased incidence of cryptococcosis was identified on southern Vancouver Island, British Columbia (BC), Canada. Clinical disease was recognized in humans, dogs, cats, ferrets, porpoises, and llamas resulting in the first multi-species outbreak of cryptococcosis (7). All animal and human isolates available for culture from BC were *C. gattii* serotype B. Since 1999 over 200 human and animal cases of cryptococcosis have been reported and the species list has been expanded to include birds and a horse (8). Cases are clustered on the east coast of the

island within the Coastal Douglas-fir (CDF) biogeoclimatic zone. Since 2001 *C. gattii* has been repeatedly and consistently isolated from soil, air and vegetation within the CDF zone (9).

Infection in animals is thought to be the result of inhalation of the airborne environmental fungi and subsequent colonization of the nasal cavity and paranasal sinuses (6, 10-12). In Australia it has been reported that dogs, cats (13) and koalas (14) can carry *C. neoformans* in their upper respiratory tract asymptotically, suggesting that nasal colonization may be much more common than clinical disease. The following study was conducted to identify the prevalence, and outcomes, of sub-clinical cryptococcosis and asymptomatic carriage of *C. gattii* in the nasal passages of dogs and cats within the CDF zone of Vancouver Island, BC.

5.2. Materials and Methods

5.2.1. Study population

Five veterinary clinics in four cities (figure 5.1) were selected as sampling sites based on caseload, identification of clinical cryptococcosis in their service area, location within the CDF region and willingness to participate. At each clinic a fixed weekday was selected where the daily caseload included both medical and surgical appointments. Owners presenting a dog or cat over six months of age to the veterinary clinic for reasons other than euthanasia or previously diagnosed clinical cryptococcosis were offered the opportunity to participate in the study.

Owners completing a consent form and a brief information sheet could elect to have both a nasal swab and a blood sample collected or one of the two. Sampling was carried out on average once every three weeks from June to December 2003.

5.2.2. Animal information

Information collected from the owner included the animal's age, sex, breed and the duration of time it had lived within the CDF zone on Vancouver Island. Owners were asked if, in the last year, their pet had shown signs suggestive of respiratory tract disease including sneezing, coughing or nasal discharge, central nervous system disease including behavioral changes, seizures, poor coordination or balance problems, skin lumps or vision changes. Owners were also asked to note other health problems observed in the last year that were not included on the list. Finally owners rated how they perceived the overall health of their pet as one of very poor, poor, good or very good. Reason for bringing the animal to the veterinarian and body weight of dogs was also recorded.

5.2.3. Animal sampling

Superficial nasal cultures were collected from unsedated animals. In dogs a single Starplex StarSwab II (Starplex Scientific, Etobicoke, ON) moistened in transport media was inserted 0.5 to 2 cm into both nasal vestibules and rotated on the mucosa. In cats a similar procedure was conducted using a Calcium Alginate Fiber Tipped Ultrafine Aluminum Applicator swab (Calgi-swab, Fisher Scientific, Toronto, ON) moistened with sterile saline (0.9% NaCl). The StarSwabs were placed in the associated transport media and the Calgi-swabs were placed in a 1.5 ml Eppendorf microcentrifuge tube (Brinkmann Instruments, Westbury, NY) containing ~0.2 cc sterile saline. Animals undergoing general anesthesia for any procedure received a second, deeper nasal swab using a Calgi-swab. The swab was rotated on the nasal mucosa of both nasal passages at approximately the level of the medial canthus of the eye.

Blood was collected using standard venipuncture technique. A minimum of 1ml of blood was collected from each animal participating in the study, allowed to clot for 15-30 minutes at room temperature and centrifuged to separate the serum.

5.2.4. Culture

Culture swabs were plated onto Bird Seed Agar and incubated at 30 °C. Plates were examined at 48 hours, then daily for seven days. *Cryptococcus neoformans* and *C. gattii* selectively use caffeic acid in the medium to produce melanin, resulting in brown colonies. Suspect colonies were transferred to Malt Extract Agar (MEA) and Canavanine-glycine-bromothymol blue (CGB) agar. *Cryptococcus gattii* turns the CGB agar cobalt blue while *C. neoformans* remain negative. Colonies growing on MEA were serotyped using capsular antibodies (Crypto-check, Iatron Laboratories, Japan). Biochemical identification was achieved using API 20 AUX strips (bioMérieux, St. Laurent, Quebec).

5.2.5. Antigen test

Samples were treated with pronase (15) prior to the use of a latex cryptococcal antigen agglutination test for the measurement of cryptococcal antigen in the sera (Cryptococcal Antigen Latex Agglutination System (CALAS); Meridian Bioscience, Inc., Cincinnati, Ohio). The CALAS test cannot identify the organism beyond the level of the genus. Animals with a titer \geq 1:2 were considered positively infected with *Cryptococcus* spp. (16).

5.2.6. Statistical analysis

When *C. gattii* was isolated from either the deep or superficial swab the animal was considered positive on nasal culture. Animals testing positive on either a nasal culture or antigen test were given an overall rating of positive for *Cryptococcus* spp. Results were stratified by species. The

owner-evaluated overall health rating was converted to a dichotomous variable where scores of very poor and poor were combined to below average; good and very good were combined to above average. Dogs were classified into two size categories based on body weight above or below 15 kilograms.

Descriptive and comparative statistics were computed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Odds ratios and 95% confidence intervals were used to evaluate the association between positive test results and animals presenting to the clinic for illness compared to routine procedures, owner perceived health status, sex, and health problems in the previous year. The Kolmogorov-Smirnov Z test was used to evaluate the normality of the distribution of continuous variables including age and duration of residence within the CDF zone. For variables with normal distribution mean values were reported and the T-test was used to compare means between positive and negative animals. Where the results of the Kolmogorov-Smirnov Z test were significant at the 5% level median values were reported and the Mann-Whitney U (MWU) test was used to compare means between positive and negative animals. The relative proportions of infected species were compared using odds ratios and 95% confidence intervals. The prevalence of positive animals in the four sampling cities was compared using a Chi-Square test. Kappa statistics were calculated to determine the agreement between the two nasal swabs and between the antigen test and nasal culture.

5.2.7. Follow-up testing

Owners of positive animals identified as sub-clinically infected with *Cryptococcus* spp. or carrying *C. gattii* in their nasal passages were asked to bring their animal to the veterinarian for follow-up testing as often as possible to a maximum of once a month. Follow-up testing was

available until October 2004. At each follow-up visit a serum sample and superficial nasal swab was collected for CALAS testing and fungal culture. Serial 10 fold dilutions were carried out on serum samples; when higher dilutions showed agglutination and dilutions were large, intermediate twofold dilutions were run in an attempt to more accurately determine an endpoint.

5.3. Results

5.3.1. Initial testing

Of serum samples collected from 84 cats, six (7.1%) had an antigen titer $\geq 1:2$ (Table 5.1). Titers ranged from 1:2 to 1:200. Superficial nasal cultures of 94 cats identified three (3.2%) animals with *C. gattii* serotype B in the nasal vestibule while deep nasal swabs from 13 cats under general anesthesia identified two (15.4%) animals with *C. gattii* serotype B in their nasal cavity. Overall 7 (7.4%) cats tested positive on one or more tests. One of the seven cats resided in the Parksville area, five in Nanaimo and one in Duncan but the proportion of cats positive on one or more tests was not statistically different between cities ($p=0.122$).

The mean age of cats testing positive on one or more test was 8 years (SD 7.0 years, range 0.7-18 years) and was not significantly different from cats who were not positive on any test performed (T-test, $p=0.54$). The median time positive cats had resided in the CDF zone of Vancouver Island was 5.0 years (range 0.5-18 years) which was not statistically different from the negative cats (median 4.8, range 0.5-16, MWU $p=0.44$).

The odds of being positive on one or more test were not statistically different for male cats relative to females (OR 1.27, 95% CI 0.27, 6.03). The odds of a positive test result was 3.33

(95% CI 0.320, 34.71) times greater in cats with an owner perceived health status of poor relative to good, however, this result was not statistically significant. The odds of a positive test result was increased in cats with a history of respiratory signs (OR 1.46, 95% CI 0.16, 13.56), central nervous system symptoms (OR 2.13, 95% CI 0.22, 20.49), skin lumps (OR 2.28, 95% CI 0.24, 22.13) and vision changes (OR 5.50, 95% CI 0.45, 67.49) in the last year relative to those without these symptoms; however, none of these results were statistically significant. The presence of other health problems in the previous 12 months was slightly protective (OR 0.68, 95% CI 0.08, 5.858) but this result was not statistically different compared to cats without other health problems. Cats testing positive on one or both tests for *Cryptococcus* spp. were less likely to have presented to the veterinarian for owner perceived illness verses routine veterinary procedures (OR 0.15, 95% CI 0.02, 1.32); however, this result was not statistically significant. The breed distribution of the positive cats consisted of five domestic shorthairs, one domestic longhair and one Himalayan.

Two of 266 (0.8%) dogs had an antigen titer of 1:2 (Table 5.1). Of 280 superficial nasal cultures *C. gattii* serotype B was isolated from three (1.1%) dogs. *Cryptococcus gattii* was not isolated from any of the 34 dogs from which a deep nasal swab was collected. Overall five (1.7%) dogs tested positive on one of the tests, no dogs were considered positive on more than one test. Four of the five dogs positive on any of the tests were from the Duncan area and the remaining positive dog was in Parksville. This difference in the prevalence of positive dogs by city was statistically significant ($p=0.03$).

The median age of dogs positive on either test was 6.6 years (range 3.4-11.6 years) which did not differ statistically from the remainder of the dogs tested (median 5.8, range 0.5-16.5, MWU $p=0.46$). Positive dogs had resided in the CDF zone for a median of 5 years (range 3.4-10 years) which was not statistically different from the negative dogs (median 4.0, range 0.5-16.0, MWU $p=0.51$). The odds of a positive test was not statistically different for males relative to females (OR 0.89, 95% CI 0.15, 5.42). The odds of a positive test result was 3.99 (95% CI 0.44, 36.01) times greater in dogs with an owner perceived health status of poor relative to good, however, this result was not statistically significant. The odds of a positive test result was increased in dogs with a history of respiratory signs (OR 4.09, 95% CI 0.43, 38.79), central nervous system symptoms (OR 1.78, 95% CI 0.21, 15.38), skin lumps (OR 1.41, 95% CI 0.15, 12.88), vision problems (OR 3.17, 95% CI 0.36, 28.13) and other health problems (OR 2.37, 95% CI 0.39, 14.52) but these results were not statistically significant. The odds of dogs presenting to the veterinarian for owner perceived illness being positive for *Cryptococcus* spp. in was 3.17 (95% CI 0.35, 28.72) times that of animals brought to the clinic for routine veterinary procedures. Dog breeds that tested positive included two Labrador Retriever crosses, one German Shepherd, one Jack Russell Terrier and one Toy Poodle. There was no significant difference in the proportion of dogs testing positive above and below 15 kilograms (OR 1.46, 95% CI 0.24, 8.92).

Cats were 10.15 times (95% CI OR 2.01, 51.31) more likely to be positive on the antigen test than dogs. Combination of the superficial and deep nasal culture results into a single dichotomous variable of positive or negative on culture revealed that cats were 4.10 (95% CI 0.90, 18.68) times more likely than dogs to carry *C. gattii* in their nasal cavity, however this result was not statistically significant at the 5% level. When the results of the antigen test and

nasal cultures were combined into a single positive or negative variable the difference between species was significant with cats being 4.42 times (95% CI 1.37, 14.28) more likely than dogs to be positive on one or both tests.

Three of 12 (25%) positive animals were positive on both the antigen test and nasal culture. Four of 12 (33%) of animals had a positive nasal culture but negative antigen test and 5 of 12 (42%) were positive on antigen test alone. A computed kappa of 0.39 (95% CI 0.28, 0.49) suggests only fair agreement between the serum antigen test and nasal culture. Forty-seven animals had both deep and superficial swabs of which 44 were negative on both cultures, one was positive on both cultures and one animal was positive on each of the deep and superficial cultures. The kappa statistic of 0.48 (95% CI 0.19, 0.76) suggests moderate agreement between the two swab techniques.

5.3.2. Follow-up testing

Twelve animals positive on initial testing had between one and four follow-up samples collected; the results for each individual animal are shown in table 5.2. Animals 1-7 are cats and 8-12 are dogs. Of the seven cats, six had a titer on one or more test dates; five of the seven had the organism isolated from their nasal cavity. One cat (#6) had the organism isolated from its nasal cavity on two of four occasions but never had a positive titer. Two cats (#'s 5 & 7) had positive titers on multiple occasions but the organism was never isolated from their nasal cavities. Four cats (#'s 1, 2, 3 & 4) had both a positive titer and organism in their nasal cavities on one or more occasions. Titers in cats ranged from 1:2 to 1:2500.

Two cats (#'s 1 & 2) progressed to clinical disease. Case 1 was a 19 year old, spayed female domestic shorthair cat presented initially to the veterinary clinic for illness. Upon initial examination the owners reported their cat to be in poor overall health with severe arthritis and regular constipation. In the previous year the owner noted that the cat had a chronic sneeze. The cat had lived on Vancouver Island all of its life. In February 2004 the cat showed central nervous symptoms including ataxia and seizures and was humanely euthanized.

Case 2 was a 15 year old, neutered male domestic shorthair cat presented to the veterinarian for annual vaccination. Upon entry into the study the owners reported the cats overall health to be very good and noted that in the previous year the cat had no symptoms suggestive of cryptococcosis. The cat had lived on Vancouver Island all of his life. Upon initial examination the cat was deemed healthy and received vaccination; although the owners reported regular sneezing. In late November 2003 the cat showed symptoms of respiratory tract infection including nasal discharge and sneezing and was started on antifungal therapy in December 2003. Ten months after initiation of therapy the CALAS titer was negative.

Of the five dogs, two had a positive titer at one or more test dates, four of the five had organism isolated from their nasal cavity. Three dogs (#'s 10, 11 & 12) had the organism isolated from their nose on initial testing but never on subsequent tests. One dog (#8) had a single positive antigen titer and organism isolated from its nasal cavity on a single occasion, but not on the same date. One dog (#9) had a titer of 1:2 on initial testing but all subsequent CALAS tests were negative and *C. gattii* was never isolated from its nasal passages. A CALAS result of 1:2 was the only titer detected in dogs.

5.4. Discussion

Identification of sub-clinical infection and nasal colonization of dogs and cats with *Cryptococcus* spp. is an important step in the characterization of the outbreak of clinical cryptococcosis on Vancouver Island. Of animals sampled within the CDF zone of Vancouver Island 7.4% of cats and 1.7% of dogs has either a positive nasal culture or antigen titer indicating that they were colonized by or infected with *Cryptococcus* spp.

On physical examination none of the animals identified as positive on either test showed signs consistent with cryptococcal disease. The most common presentations of feline and canine cryptococcosis include respiratory, central nervous system, dermal or ocular symptoms (17-19). Owners of positive dogs and cats reported a slightly increased prevalence of symptoms suggestive of cryptococcosis within the previous 12 months, however these results were not statistically significant. The odds of an animal testing positive was increased in animals considered to be in below average health by their owners, however, this result was not statistically significant and positive animals were equally likely to have presented to the veterinarian for routine procedures as for owner-perceived illness.

Cryptococcus gattii serotype B was isolated from 4.2% and 1.1% of cats and dogs, respectively. Although superficial nasal swabs were assumed to confer good agreement with a nasal flush in koalas (14) numerical results were not reported and extrapolation of this assumption across species should be made with caution. In this study there was a wide confidence interval with only moderate agreement between the two swab techniques. The lack of pattern in the disparity between deep and superficial swabs suggests that both samples may underestimate true nasal

colonization. Correspondingly there was only fair agreement between the antigen test and nasal culture. The kappa statistic is highly dependent on the true prevalence of disease in the population and where prevalence approaches one or zero kappa is sharply reduced (20). Further studies on a cohort of animals with a higher prevalence of colonization and infection are required to evaluate test agreement more meaningfully.

Of the animals positive on one or more tests 33% had a positive nasal culture without a positive antigen titer. Studies of presumed healthy animals in Australia recovered *C. neoformans* and *C. gattii* from cats, dogs (13) and koalas (14, 21). Based on the lack of cryptococcal antigen or pathology of the nasal cavity these studies concluded that *Cryptococcus* spp. can colonize the nasal passage of animals without an associated local or systemic infection. In contrast 25% of animals tested in BC that had *C. gattii* in their nasal cavities also had antigen in their serum suggesting sub-clinical infection versus nasal colonization. Forty-two percent of animals positive on any test had an antigen titer without a positive nasal culture. The CALAS test has been reported to have high specificity in diseased cats and dogs (22, 23) making false positive reactions unlikely; however, effectiveness of the CALAS test in asymptomatic animals has not been evaluated. Furthermore, the sensitivity of nasal culture in colonized animals is unknown and it is possible that the organism was missed during the nasal swab or that the source of antigen was not in the nasal passage.

Overall cats had significantly greater odds of testing positive on either culture or antigen test or antigen test alone when compared to dogs. Cats had increased odds of carrying *C. gattii* in their nasal cavity but this result was not statistically significant. Given the low number of positive test

results this study may lack the power necessary to identify a statistical difference.

Cryptococcosis is the most common systemic mycoses of cats (19) and clinical disease has been reported with equal or greater frequency in cats than in dogs (18). Likewise, on Vancouver Island the reported number of clinical cases in cats outweigh that of dogs (8).

Previously published studies on risk factors for animal cryptococcosis focus only on clinical cases. Cryptococcal disease has been reportedly been more common in young large dogs (12, 24) and male cats (10, 25, 26), potentially for behavioral reasons. This study failed to identify sex, age or size of dog as statistically significant factors for asymptomatic infection or colonization by the organism. Elsewhere environmental exposure to infectious organism is considered a risk factor for cryptococcal disease (12, 27). While *C. gattii* has only been identified within the CDF zone on Vancouver Island (9) the duration of time dogs and cats lived within the region was not a risk factor for asymptomatic infection or colonization. Previously reported risk factors may be restricted to animals that become clinically ill and not apply to asymptomatic or otherwise healthy animals. Subsequent investigations into risk factors for asymptomatic infections should include a larger sample size to increase the study power. Because the distribution of cat and dog breeds within the study population is unknown the effect of breed cannot be evaluated.

All of the sampling clinics lie within the CDF zone but the highest proportions of positive animals were in Duncan and Nanaimo which are located in the center of the testing area. It is interesting to note that no animals tested positive in Victoria which lies on the southern most extreme of the CDF zone. In Australia koalas have been used to successfully identify geographic

areas with a high-grade presence of *C. gattii* in the environment (21). Further sampling of dogs and cats at the edge of the CDF zone in combination with environmental testing may identify companion animals as a similar sentinel.

Follow-up testing of positive animals revealed that dogs and cats can clear the organism, remain sub-clinically infected or progress to overt disease. *Cryptococcus gattii* was initially isolated from the nasal cavity of three dogs without antigenemia; however the organism was never recognized on subsequent testing. A single cat had *C. gattii* isolated from the nasal cavity on two of four visits while cryptococcal antigen was never found in its serum. These results suggest the nasal passages of animals residing within a region where environmental *C. gattii* is present, may be transiently colonized by the organism without rapid progression to infection. Failure to re-isolate the organism from any of the dogs suggests that the presence of the organism in the nose is relatively brief; or that the swab technique is not sufficient to capture the organism at each sampling interval. *Cryptococcus gattii* was isolated twice from the cat suggesting better recovery of the swab, more persistent colonization or re-exposure of the animal to airborne fungi. As the CALAS test detects only circulating antigen, extension of infection beyond the nasal mucosa is required before the test can be positive. Factors mediating progression from colonization to infection eliciting antigenemia are unknown although dose response, concurrent disease or other forms of immunosuppression have been suggested (3, 21).

One dog had an antigen titer of 1:2 on initial testing but not on any follow-up visits, *Cryptococcus* spp. was never isolated from its nose. Two cats had positive titers on more than one occasion but the organism was never isolated from the nasal cavity. The sensitivity of

superficial nasal culture in animals is unknown and it is possible that the organism was missed during the nasal swab, or that the source of antigen was not in the nasal passage. As the effectiveness of the CALAS test has not been evaluated in asymptomatic animals false positive results cannot be ruled out. One dog had a low titer with no culture on initial testing; on follow-up examination *C. gattii* was isolated from the nasal passage but the CALAS test was negative, disparities potentially elicited by the aforementioned limitations of the diagnostic tests. These four animals with antigenemia on one or more occasion were all CALAS negative at the end of the study, between five and 11 months after the date of the last positive CALAS test. These results suggest that the animals may have cleared the infection. Demonstration of cryptococcal antigen in serum or cerebral spinal fluid implies infection with the organism and a titer of 1:2 has been reported to be clinically relevant in cats (16). Asymptomatic infection has been proposed to be a self limiting condition in koalas but individuals with low positive titers may harbor foci of infection that could reactivate (21).

Four cats had both antigenemia and *C. gattii* in their nasal cavity; two of these progressed to clinical disease. Both animals had antigen titers greater than other cats in the study and titer values increased over the sampling period. The highest titer observed in a cat not showing clinical signs was 1:32. In a cohort study of koalas in Australia all clinical animals documented in the study had antigen titers $\geq 1:128$ and an increased incidence of nasal colonization; while animals without clinical symptoms had titers $\leq 1:64$ (21). The results of this small study suggest that asymptomatic animals with a titer equal to or less than 1:32 clear the infection while those with a higher titer go on to become diseased. Further investigation into the relationship between

asymptomatic colonization and clinical disease is warranted as clinical cases in BC have been diagnosed with titers as low as 1:2 (23).

The findings of this study demonstrate the need for a better understanding of sub-clinical infection and nasal colonization of *C. gattii* in companion animals. In areas of Australia where environmental exposure of koalas to *C. gattii* is high, sub-clinical infection is relatively common while progression to clinical disease is rare (21). Given the recent emergence of the organism in southwestern BC more information on environmental load, variables influencing exposure and risk factors for progression from colonization to clinical disease is warranted.

Table 5.1: Positive animals and odds ratios for cats relative to dogs tested on Vancouver Island, BC, Canada

	Feline	Canine	OR	95% CI
Any test	7/95	5/283	4.42	1.37, 14.28
CALAS	6/84	2/266	10.15	2.01, 51.31
Total culture	4/94	3/280	4.10	0.90, 18.68
Superficial	3/94	3/280	-	-
Deep	2/13	0/34	-	-

CALAS: Cryptococcal Antigen Latex Agglutination System

Table 5.2: CALAS titer and results of nasal *C. gattii* culture on follow-up testing

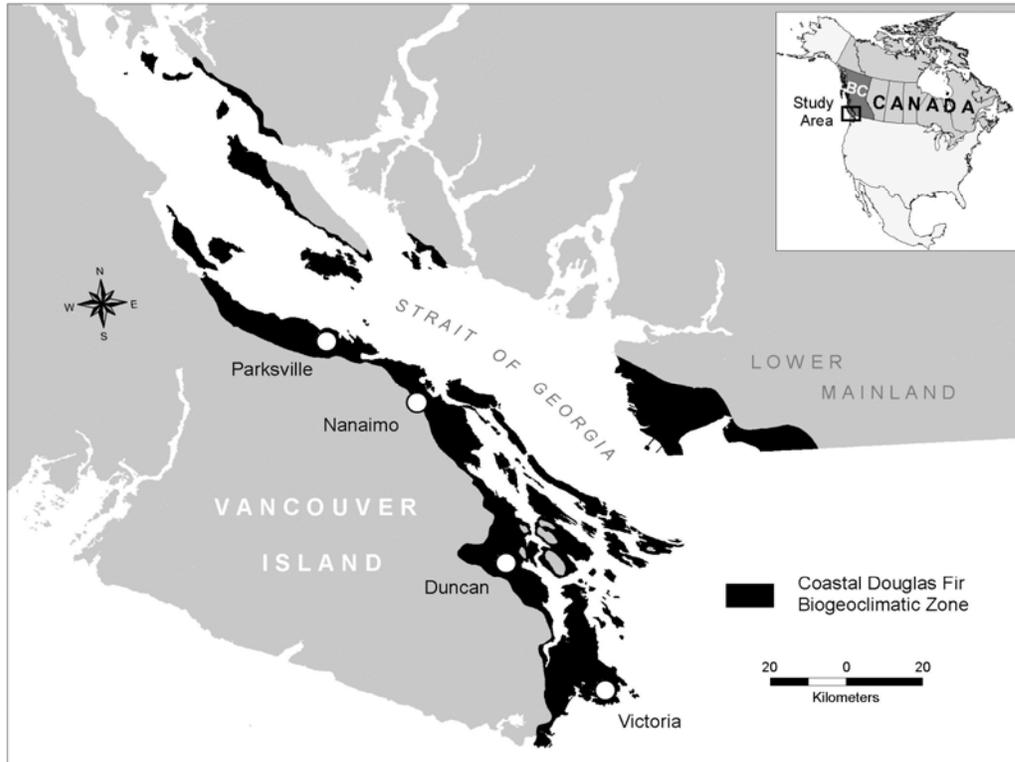
Date	Test	Animal ID											
		1*	2†	3	4	5	6	7	8	9	10	11	12
Aug-03	CALAS	1:200	1:64	1:2	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Culture	pos	pos	pos	NT	NT	NT	NT	NT	NT	NT	NT	NT
Sep-03	CALAS	1:2	1:50	NT	1:2	NT	NT	NT	1:2	1:2	0	0	NT
	Culture	pos	pos	NT	pos	NT	NT	NT	neg	neg	pos	pos	NT
Oct-03	CALAS	1:200	NT	0	NT	NT	NT	NT	0	0	0	NT	0
	Culture	pos	NT	neg	NT	NT	NT	NT	pos	neg	neg	NT	pos
Nov-03	CALAS	1:2500	1:1024	1:16	NT	1:32	0	NT	NT	NT	NT	0	0
	Culture	NT	neg	neg	NT	neg	pos	NT	NT	NT	NT	neg	neg
Dec-03	CALAS	1:2500	1:500	1:2	NT	1:2	0	1:2	0	0	0	0	NT
	Culture	pos	pos	neg	NT	neg	pos	neg	neg	neg	neg	neg	NT
Jan-04	CALAS	NT		NT	1:2	NT	NT	1:2	NT	NT	NT	NT	NT
	Culture	NT		NT	pos	NT	NT	neg	NT	NT	NT	NT	NT
Feb-04	CALAS			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Culture			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Mar-04	CALAS			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Culture			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Apr-04	CALAS			NT	NT	1:2	0	1:2	NT	NT	NT	NT	NT
	Culture			NT	NT	NT	neg	NT	NT	NT	NT	NT	NT
May-04	CALAS			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Culture			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Jun-04	CALAS			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Culture			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Jul-04	CALAS			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Culture			NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Aug-04	CALAS			0	NT	NT	NT	NT	0	0	0	0	NT
	Culture			neg	NT	NT	NT	NT	neg	neg	neg	neg	NT
Sep-04	CALAS			NT	NT	NT	0	NT	NT	NT	NT	NT	NT
	Culture			NT	NT	NT	neg	NT	NT	NT	NT	NT	NT
Oct-04	CALAS			NT	NT	0	NT	0	NT	NT	NT	NT	NT
	Culture			NT	NT	neg	NT	neg	NT	NT	NT	NT	NT

NT = not tested, neg = negative

* died February 2004

† began antifungal therapy December 2003

Figure 5.1: Location of sampling clinics (clear circles) and distribution of the Coastal Douglas Fir Biogeoclimatic zone on Vancouver Island, BC, Canada.



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6. *Cryptococcus gattii* in horses and wildlife of Vancouver Island, British Columbia, Canada

6.1. Introduction

Since 1999, *Cryptococcus gattii*, a species now distinct from *C. neoformans* (1), has emerged as an important pathogen of humans and animals in southwestern British Columbia (BC) (2-5). Previously only *C. neoformans* had been isolated from animals or humans in Canada and *C. gattii* was thought to be restricted to the tropics and sub tropics (6, 7). Clinical illness has been identified in humans and numerous animal species in BC including cats, dogs, ferrets, llamas, porpoises, domestic birds and a horse. Cases to date have been clustered on the east coast of Vancouver Island; largely within the coastal Douglas fir (CDF) biogeoclimatic zone (8). *Cryptococcus gattii* has been routinely isolated from soil, air and vegetation within the CDF zone since 2001 (4, 9).

Given the airborne nature of this organism, it may be assumed that many species residing within endemic areas are exposed, but infection has been largely unnoticed.

Asymptomatic carriage of *C. gattii* has been recognized in companion animal species of BC; presumably as a result of contact with airborne infectious material (8, 9).

Environmental exposure and asymptomatic colonization of the respiratory tract has been proposed to be much more common than clinical disease (10, 11), however, variables influencing the initiation of infection remain unclear. The prevalence of exposure of humans and animals in BC is unknown.

The objective of this study was to identify terrestrial mammalian wildlife species and horses that have been exposed to or infected with *C. gattii* on Vancouver Island, BC. Horses were selected because, on Vancouver Island, they are housed predominantly outdoors, are dispersed throughout the endemic area, are susceptible to the organism and public concerns regarding equine cryptococcosis facilitates sampling. Wild mammal species were targeted because samples were accessible and the majority of domestic cases on Vancouver Island were mammals.

6.2. Materials and Methods

6.2.1. Wildlife sampling

Between February and August 2004, a deep swab of the nasal mucosa using a Starplex StarSwab II (Starplex Scientific, Etobicoke, ON) and lung tissue, where possible, were collected for fungal culture. Sources of live and dead animals included wildlife rehabilitation facilities, veterinarians, biologists and registered trappers. Any mammalian species live-trapped or killed and known to reside in the CDF zone on Vancouver Island between January 1999 and August 2004 were eligible for inclusion in the study. Species and approximate life stage were recorded along with the location of animal capture when available.

6.2.2. Equine sampling

All BC veterinary medicine association (BCVMA) registered veterinary clinics servicing equine clients within the region where clinical cryptococcosis cases had been identified were contacted for participation. Veterinarians were given maps of their service area that included sites where *C. gattii* had been isolated from the environment (Bartlett,

unpublished) and an associated 10 km zone around the positive sample locations.

Practitioners were asked to provide names of equine clients residing within the 10 km zone that would be interested in participating in the study.

Between July 24 and August 9, 2004 samples were collected from horses residing in identified buffer zones. Material collected included a swab of the nasal mucosa using StarSwab II (Starplex Scientific, Etobicoke, ON) moistened in transport media, inserted 10-15 cm into both nasal vestibules and rotated on the mucosa. A minimum of 5 ml of blood was collected from each animal participating in the study using standard venipuncture technique. Blood was allowed to clot for a minimum of 30 minutes and centrifuged to separate the serum.

Data collected on each horse tested included age, breed, underlying health problems, duration lived at sampling location and on Vancouver Island, average time spent outside per 24 hour period, source of hay and if hay was fed on the ground or in a feeder. The difference in age, duration of residence on current property and Vancouver Island between positive and negative horses was evaluated using the Mann-Whitney U test. The relative number of horses fed on or off of the ground, local or imported hay and housed outdoors or both in and out were compared using the Fishers exact test.

6.2.3. Laboratory analysis

Culture swabs were plated onto Bird Seed Agar and incubated at 30 °C for 48 hr. Plates were checked for growth daily for ten days before being regarded as negative. Colonies conforming to cryptococcal morphology were identified and serotyped using

agglutinating antibodies (Crypto-check, Iatron Laboratories, Tokyo, Japan). Lung tissue was splayed on a sterile surface and dissected to allow access to the interior surface using a scalpel blade sterilized by alcohol dip and flaming. Internal and external surfaces were swabbed using a cotton-tipped applicator (Puritan, Fisher Scientific). The applicator was rolled across a differential agar (Bird Seed Agar) and a rich nutrient agar (Saboraud Dextrose Agar, BBL). Agar plates were incubated and checked for growth as above.

Serum samples were treated with pronase (12) prior to the use of a latex cryptococcal antigen agglutination test for the measurement of cryptococcal antigen in the sera (Cryptococcal Antigen Latex Agglutination System (CALAS); Meridian Bioscience, Inc., Cincinnati, Ohio). The CALAS test cannot identify the organism beyond the level of the genus. Animals with a titer $\geq 1:2$ were considered positively infected with *Cryptococcus* spp.

6.3. Results

6.3.1. Wildlife sampling

Nasal swabs were collected from 91 individuals representing 14 species; 19 living harbor seals (*Phoca vitulina*) and 72 post-mortem samples. A list of all species, the respective number of isolates and age categories of animals sampled are presented in table 6.1.

Lung tissue was available for culture from 68 animals representing 13 species.

Cryptococcus gattii was isolated from the nasal swab of two Eastern Grey Squirrels (*Sciurus carolinensis*) trapped at the same location in the city of Duncan, BC. Lung tissue was available from one of these squirrels and fungal culture of the tissue yielded no *Cryptococcus* spp.

6.3.1. Equine sampling

Nasal swabs and serum samples were collected from 260 horses residing within 10 km of a site where *C. gattii* had been isolated from the environment. No horses were positive on the antigen test, but the organism was isolated from the nasal passages of four horses. All positive horses resided in the area of Duncan, BC with one horse directly adjacent to the trapping site of the two positive squirrels.

All horses, except for one, had lived on Vancouver Island their entire lives. This horse had moved to the island approximately six months prior to testing and resided only in the Duncan area during that time. The median duration of residence on Vancouver Island was 5 years (minimum 3 months, maximum 9 years) for the positive horses and 8 years (minimum 3 months, maximum 32 years) for the negative horses; a difference that was not statistically significant ($p=0.198$). The median duration of positive horses living on the property where testing occurred (2.25 years, minimum 3 months, maximum 6 years) did not differ significantly from that of negative horses (3 years, minimum 3 months, maximum 23 years). The four positive horses were 4, 6, 9 and 10 years of age; the median age of positive horses (7.5 years) did not differ significantly from that of horses without organism in their nose (12 years, minimum 3 months, maximum 35 years, $p=0.202$). The proportion of positive horses fed on the ground did not differ statistically from the negative horses ($p=0.264$). None of the positive horses had any owner reported illness or historical medical problems.

6.4. Discussion

Recent investigation into subclinical infection in companion animals identified 4.3% of cats and 1.1% of dogs residing within the CDF zone of Vancouver Island had *C. gattii* in their nasal cavity (8). The organism was present on 1.5% of nasal swabs collected from horses and 2.2% of swabs collected from wild mammals in this study. Identification of *C. gattii* in the nasal passages of animals is likely the result of environmental exposure. Lung tissue was available from one of the positive squirrels but the organism was not isolated. Cryptococcal antigen was not identified in serum samples collected from any of the four horses suggesting nasal colonization and not systemic infection. Both grey squirrels were trapped and humanely euthanized on private property as the species is considered an invasive alien in this area; both were presumed to be healthy. On gross post-mortem examination no lesions were observed suggestive of clinical cryptococcosis or other diseases. Failure to identify pathology or systemic infection in an individual suggests nasal colonization resulting from environmental exposure and not clinical infection. Without isolation of the organism from a normally sterile site, histological examination of tissue from the nasal cavity, or serum upon which to run a cryptococcal antigen test, it is difficult to make inferences on the status of the organism within the respiratory tract; however it appears that the squirrels were colonized by and not infected with the organism as per the horses.

Both positive squirrels and all four horses were from the same geographic location. The city of Duncan is central in the region in which clinical cryptococcosis cases have been reported (13) and a cross-sectional study in dogs and cats identified Duncan to have a

higher proportion of colonized or sub-clinically infected animals (8). Environmental *C. gattii* has not been isolated ubiquitously on Vancouver Island (4) and investigation into environmental *C. gattii* identified increased concentration of organism in soil samples collected from the Duncan area relative to most other parts of Vancouver Island (Bartlett, unpublished).

As the two squirrels were the only wildlife species submitted from this region it is difficult to draw conclusions regarding the relative role of location compared to species of wildlife. The remaining negative squirrels were submitted from Victoria (n=13) and Salt Spring Island (n=1). Further investigation into nasal colonization of wildlife species within a region where the environmental organism has been quantified is an important step in the understanding of the prevalence of the organism in wild populations. In Australia it has been proposed that heavily colonized or infected koalas may contaminate previously culture negative vegetation (14). Eastern Grey Squirrels were introduced to Vancouver Island in the Victoria area; their northward expansion may facilitate transmission of *C. gattii* to regions currently free from of the organism.

Over 74% of wild mammals sampled in this study were collected from wildlife rehabilitation facilities. While younger animals are often over represented, this sampling technique is an inexpensive way to collect samples from multiple species in a short period of time. It is important however, to consider the effect of a young sample population on the results; younger animals will have had a shorter duration of exposure

and, given the long incubation period of *Cryptococcus* spp., younger animals may not manifest signs of clinical disease at the time of sampling.

Failure to culture the organism from the nasal cavity of any living wildlife may be influenced by the inability to sample with the same intensity; swabs collected from living animals were more superficial than those collected post-mortem. *Cryptococcus gattii* has been repeatedly isolated from the nasal cavity of living wild and domestic animals however there are conflicting results concerning the agreement between deep and superficial nasal swabs (8, 10). Standardization of sampling techniques is important in cross species studies; given the lack of data for agreement between the sampling techniques in wild mammals it should be noted that samples collected from living animals differed from those samples collected post-mortem. The sensitivity of nasal culture in animals is unknown and it is possible that the organism is missed during a nasal swab, or that infection is present in a site other than the nasal passage. Antigenemia but failure to isolate the organism from the nasal cavity has been reported in asymptomatic animals (8) and clinical cases (Duncan, unpublished). The methodology used in this study may not be sufficient to identify exposure or infection in all animal samples.

The prevalence of nasal colonization observed in horses in this study is similar to that of companion animals; however, there has been only one case of clinical *C. gattii* infection diagnosed in a horse on Vancouver Island to date (Raverty, unpublished). This discrepancy may reflect differing species susceptibility to clinical disease or failure to diagnose clinical cases because they are not being seen by veterinarians or the diagnosis

is being missed. In companion animals, feline cases outnumber disease in canines by over 50% suggesting a variation in species susceptibility (Duncan, unpublished). Horses may be less susceptible to clinical disease than either dogs or cats.

Age of horse, breed, underlying health problems, duration at sampling location and on Vancouver Island, average time spent outside per 24 hour period, source of hay and feeding methods were not identified as statistically significant risk factors for nasal colonization with *C. gattii* in this study. It is important to note however that there were not enough positive horses in this study to make significant conclusions regarding risk factors. While geographic location relative to environmental organism is likely the most significant variable influencing exposure, it is important to identify other risk factor, if present, such that owners and veterinarians can attempt to mitigate risk where possible.

The recent emergence of *C. gattii* in western Canada dictates the need to identify the population at risk. Wildlife and horses, by virtue of living outdoors all or most of the time and therefore being constantly exposed to airborne organism, may be a better ‘environmental indicator’ of human risk than companion animals. The collection of nasal swabs from wildlife species or horses residing within endemic regions of BC may be an inexpensive way to survey the environment and quantify environmental load. To date only one horse has been diagnosed with *C. gattii* infection on Vancouver Island (Raverty, unpublished). The impact of environmental *Cryptococcus* spp. on wildlife of BC remains largely unknown; further investigation is warranted.

Table 6.1: Species, age and number of wild animals tested for *Cryptococcus gattii*

Species	Number	Age	
		Adult	Juvenile
Harbor seal (<i>Phoca vitulina</i>)	22	0	22
Cottontail rabbit (<i>Sylvilagus</i> spp.)	20	4	16
Black Tailed Deer (<i>Odocoileus hemionus</i>)	15	4	11
Eastern Grey Squirrel (<i>Sciurus carolinensis</i>)	16	12	4
North American river otter (<i>Lutra canadensis</i>)	6	6	0
Red squirrel (<i>Tamiasciurus hudsonicus</i>)	2	1	1
Deer mouse (<i>Peromyscus maniculatus</i>)	2	2	0
Raccoon (<i>Procyon lotor</i>)	2	0	2
Black Bear (<i>Ursus americanus</i>)	1	0	1
Domestic rabbit (<i>Oryctolagus cuniculus</i>)	1	1	0
Marten (<i>Martes americana</i>)	1	1	0
Rat (<i>Rattus rattus</i>)	1	1	0
Vancouver Island marmot (<i>Marmota vancouverensis</i>)	1	1	0
Vancouver Island wolf (<i>Canis lupus</i>)	1	1	0

6.5. References

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7. Discussion

The emergence of *Cryptococcus gattii* in Canada contradicts previously accepted ecology of the organism. Variables influencing clinical disease caused by this ‘new’ pathogen were largely unknown and prompted physicians, veterinarians, microbiologists and epidemiologists to explore the changing picture of cryptococcosis in British Columbia. Information fundamental to understanding *C. gattii* infection includes knowledge of the population at risk, characterization of the infecting organism, spatial and temporal distribution of disease and a better appreciation for variables driving emergence of the pathogen in this new environment. The previous chapters address some, but not all, of the problems related to the emergence of *C. gattii* in Canada.

Through record reviews of veterinary laboratories and human diagnostic services a series of presumed or confirmed *C. gattii* cases was compiled and presented in chapter two; this data reflects the general pattern of host, spatial and temporal distribution of clinical disease in 1999-2003. During this time period there was an increase in the annual number of animal cases diagnosed while the human cases plateaued in the later years; no seasonality was observed. There were almost 75% more animal cases than human cases even though it was hypothesized that animal cases are more likely to go undiagnosed or unreported when compared to humans. Animal cryptococcosis cases were identified on Vancouver Island prior to 1999 suggesting the organism may have emerged in the region prior to its identification as a causative agent for human disease. This information implies that animals, by virtue of increased case counts and, potentially, earlier onset of

disease, may serve as a good sentinel for human cryptococcosis infection and their role in characterizing the emergence of *C. gattii* in Canada should be endorsed.

Chapter three further explored host characteristics influencing clinical disease and outcomes of canine and feline cases. There were 50% more feline than canine cases and disease appeared more commonly in middle aged cats and younger dogs. There was no sex predilection for either species. The primary system involved was most commonly respiratory, followed by central nervous system (CNS) in both cats and dogs. There was, however, a higher percentage of CNS disease in dogs relative to cats, and cats were much more likely to have subcutaneous or dermal masses relative to dogs. Multivariate survival analysis identified only the presence of neurological symptoms as a statistically significant predictor of mortality; those animals exhibiting CNS symptoms were over four times more likely to die than those never showing neural signs. This information provides a summary of information from which veterinarians can make clinical decisions and characterizes the disease in companion animals of western Canada.

A case-control study, presented in chapter four, was used to identify host and environmental risk factors for clinical *C. gattii* infection in dogs and cats on Vancouver Island. Recognized risk factors included residing within 10 kilometers of commercial logging or soil disruption, travel on Vancouver Island in the previous year, increased percentage of time spent outside a 10 kilometer radius of the home, increased animal activity level, owners hiking or visiting botanical gardens and knowing other cryptococcosis cases. Taken together this data suggests that where an infectious agent is

not uniformly distributed, individual risk increases when the organism is re-distributed through large scale environmental disturbance, or when the animal has increased opportunities for exposure through travel or activity level. Identification of risk factors is required before risk can be mitigated in any way; characterization of these factors for companion animals provides clinicians and owners with information that can be used to formulate diagnostic and prevention plans. In the bigger picture these findings provide valuable information on the relationship between the environmental reservoir and disease risk. Expansion of this study to look at risk factors for human disease, as well as evaluation of risk mitigation strategies (i.e. ongoing environmental fungicide trials) are necessary prior to development or implementation of any large scale control measures.

Having characterized much of the available clinical data, the next step was to look at asymptomatic animals in attempt to determine the role of exposure on clinical disease. Serum samples and material for fungal culture were collected from dogs, cats, horses and terrestrial mammal species residing within the region where clinical cases had been diagnosed. Nasal colonization was identified in squirrels, horses, dogs and cats. Most of the animals sampled had no signs of systemic infection as determined by post-mortem examination or cryptococcal antigen testing. This suggests environmental exposure and not infection with the organism.

A greater proportion of positive animals were identified in the area of Duncan, BC on the south east coast of the island. This trend was observed consistently through companion animal, equine and wildlife sampling. The local health area encompassing the city of

Duncan has had the greatest per capita number of human cases and relatively high numbers of both canine and feline cases. Environmental testing has also isolated high concentrations of organism from regional soils. Examination of asymptomatic animals may be a valuable, indirect measure of environmental organism load. Better understanding of nasal colonization of animals by *C. gattii* is required before such a sampling technique could be employed.

From a clinical standpoint one of the most important questions warranting further investigation is into variables influencing the transition from asymptomatic nasal colonization to clinical disease. Asymptomatic infection, defined as the presence of cryptococcal antigen in the bloodstream in the absence of clinical symptoms, was identified in a small number of dogs and cats. Fourteen months of follow-up testing of asymptomatic animals revealed that animals can progress to clinical disease, remain sub-clinically infected or clear the organism. Given that environmental *C. gattii* is unlikely to disappear from the region, and that its airborne nature can expose a large population of individuals to infectious organism, it seems that an important, but poorly understood, part of the pathogenesis of disease is how the organism can infect some exposed individuals but not others. Given the low prevalence of nasal colonization observed in all species tested in this research it would be difficult to obtain adequate samples from field data to explore this problem. Experimental infection in animals under standardized conditions is necessary to effectively address this question.

The results of this research emphasize the role of animals in the study of emerging disease. Identification and reporting of animal cases assisted public health authorities and microbiologists to define a geographical area in which to focus investigation efforts. Recent identification of animal *C. gattii* cases in Washington, USA (M. Leslie, pers. com., 2005) dictates the need for research into the pattern of disease as it spreads to a new country. Information gained through the Canadian investigation will be central in the development of surveillance strategies in the United States and elsewhere.

Given the rate of change in human ecology worldwide it may be assumed that emerging infectious diseases of man and animals will remain a concern for centuries to come. Before we can effectively prevent, or even begin to understand new or re-emerging infection we must take a step back and examine the disease from the angles of host, agent and, increasingly, the environment. *Cryptococcus gattii* has afflicted less people and animals than vehicular traffic on the island's major roadways; however its emergence in Canada is unprecedented and may reflect the changing social and ecological environment in the region. Without a better understanding of variables driving emergence we will be lack the tools necessary to effectively manage future diseases. In the words of Louis Pasteur, 'the microbe is nothing, the terrain is everything'.

Appendix 1: Interview Form

Case name: _____
Animal Name: _____
Species: _____
Address: _____
Telephone: _____
Case Number: _____
Date of Diagnosis _____
Status of animal _____

Section A. RESPONDER

A.1 Who is responding to this interview?

- Primary care giver
 Other (Who _____)

Section B. DEMOGRAPHIC INFORMATION

The following are general questions about you and your animal:

B.1. What is animal's date of birth? ____ / ____ / ____ (d/m/y)

B.2 Age years: _____

B.3 Animals Sex:

- Intact Male Neutered male
 Intact Female Spayed female Unknown

B.4 Species:

- Dog (Specify Breed) _____
 Cat (Specify Breed) _____

B.5 How many did you have during the 6 month exposure period?

Dog(s) _____
Cat(s) _____
Bird(s) _____
Ferrets(s) _____
Other _____

B.6 Are you living at the same address as you were during the 6 month exposure period?

- Yes No

B.7 If yes, what is your present home address? (where animal physically lives)

Street _____

City _____

Postal Code _____

B.8 If no, What was your previous address where you lived with your pet?

Street _____

City _____

Postal Code _____

B.9 Estimate the following in terms of percent of the animals typical 24 hour day:

Confined to the house _____ h = _____ %

Confined to a cage/kennel outside _____ h = _____ %

Confined to a fenced-yard _____ h = _____ %

Outside on controlled walks _____ h = _____ %

Outside on 'off leash' walks _____ h = _____ %

<p>WHERE?</p>

Allowed to roam outside freely _____ h = _____ %

B.10 Overall, what percent of a 24 hour day does the animal spend outdoors? _____ %

B.11 In an average week what proportion of time does your pet spend outside of an area 10km (6 miles) around your home? _____ %

B.12 How many years has your pet/animal lived in municipality of residence?

_____ # years Don't know

B.13. Six months before your pet became ill with CD _____ to _____

Did your pet live within less than a mile of a:

	Yes	No	Not Sure	Refused
Wooded area				
Farm <i>If YES, what type of farm</i>				

B.14 Has your pet lived within 10 km (6 miles) of an area of soil disturbance (excavation, building, pipe laying etc) in the 6 months prior to illness:

	No	Yes, where?	Excavation by whom?
(_____ to _____)			

B.15 Has your pet lived within 10 km (6 miles) of an area of logging or vegetation clearing during the 6 months prior to illness:

	No	Yes, where?	By whom?
(_____ to _____)			

Section C. ACTIVITIES

C.1 Was your pet involved in any of the following activities 6 mo. before becoming ill?

Activity	Yes	No	DK	refused
Hunting (as a predator)				
Digging in soil				

C.2 Do you grow, or does your pet have contact with eucalyptus trees? Yes No

C.3 Have you or your pet used any of the following types of products that may have contained eucalyptus?

	Yes	No	Not Sure	Refused
Oil				
Soap				
Shampoo				
Air freshener				
Other, specify				

C.4 Were eucalyptus cuttings brought into the pet's home (for flower arrangements etc)

Yes No

C.5 Was wood (eg for burning etc) or other vegetation brought into the home?

No Yes What type? _____ Source? _____

C.6 Six months prior to your animals diagnosis with CD were you involved in any of the following activities:

	Yes	No	Pet accompanied	Not Sure
Construction				
Outdoors House/building repair				
Cleaning bird roosts				
Handling birds (pigeons or other)				
Digging soil				
Cutting/chopping wood				

Pruning or branch clean up				
Hiking If YES, Times/year _____ Where hiked				
Visit a botanical garden If YES, where				
Gardening <i>IF YES GO TO C.7 ELSE C.8.</i>				

C.7 Did you garden:

Year round Spring Summer Fall Winter

C.7.1 How many days a week did you garden 3 months prior to your animal's illness?

1-3 4-5 6-7 Don't know

C.7.2 Did you do your gardening:

Indoors (greenhouse or in the house) Outdoor Both Don't know

C.7.3 What kind of garden did you have (Mark all that apply)?

Vegetable Flower Tree Other (specify) _____ Don't know

C.7.4 Did your animal come in contact with:

Compost Y N Commercial fertilizer Y N
Bark Mulch Y N Purchased topsoil Y N

C.8 Travel History: Where have you traveled with your pet...

	In the last year
On Vancouver Island	
Off of Vancouver Island	

C.9 Do you know of anyone or another animal that has been diagnosed with cryptococcosis?

Yes No

Person Animal Who was it? _____

Don't know Refused When did that case occur? _____

Section D. PETS MEDICAL INFORMATION

D.1 Who is your regular veterinarian?

Vet's Name _____
 Address _____

D.2 In the past year, has your pet seen more than one veterinarian:

At the same _____ or different clinics _____
 Vet's Name _____
 Address _____

Vet's Name _____
 Address _____

D.3 How many veterinarians did your pet see before CD was diagnosed? _____

D.4 If they own/have in the house more than 1 animal

D.4.1 Have any other of your animals been sick?

Yes No Don't know Refused

D.4.1.1 If Yes, What type of illness _____

D.4.1.2 Did they visit a vet?

Yes No Don't know Refused

Section E. PETS MEDICAL HISTORY

E.1 Has your pet ever been diagnosed by a veterinarian with any of the following medical conditions **before** he/she had CD?

	Yes	No	Not Sure	Refused
Pneumonia				
Chronic lung problems				
Diabetes (if YES do you give insulin)				
Skin infections				
Ear infections				
Arthritis				
Liver disease				
Kidney disease				
Cancer, specify IF YES, what treatment: Chemo; surgery; DK				
Other fungal infections (list)				
Allergies If Yes, What treatment				

Immunocompromising condition (FIV, FeLk)				
Skin growths or lumps				
Other, specify				

E. 2 Has your pet been vaccinated in the past year? Yes No

Date of last vaccination?

E.3 What has your animal been vaccinated for?

	Vaccine	Yes	No	DK
Dogs	DA2PP			
	Rabies			
	Kennel cough			
	Lyme disease			
	Other			
Cats	Feline leukemia			
	Feline Distemper (Panleuk)			
	Feline Respiratory disease			
	Rabies			
	Feline Infectious Peritonitis			
	Other			

E.4 In the year before the diagnosis of CD was your pet on steroids for health problems?

Yes No Don't know Refused

E.5 When did your pet's symptoms first start? (mm/yy) _____

E.6.1 Was your pet treated for CD: Yes No

E.6.2 How many weeks or months did you give him/her the medication(s)?

	Refused	# weeks	# months
Amphotericin B			
Ketoconazole			
Fluconazole			
Itraconazole			
Other <i>If YES, please specify:</i>			

E.7 Has your pet received any medications in the last year?

Yes No

If YES date, condition treated and drug used.

