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

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Since 1999, Cryptococcus gattii has emerged as an important pathogen of humans and animals in British Columbia, Canada. Nasal swabs and serum samples were collected from dogs and cats residing within the Coastal Douglas Fir biogeoclimatic zone on Vancouver Island, where clinical cases have been reported. Deep and superficial nasal fungal cultures of 280 dogs and 94 cats identified four (4.3%) cats and three (1.1%) dogs with C. gattii serotype B in their nasal cavity. Serum samples collected from 266 dogs and 84 cats identified six (7.1%) cats and two (0.8%) dogs with a positive cryptococcal antigen titer. Overall cats were 4.4 times more likely than dogs to be positive on one or both tests. Identification of sub-clinical infection and nasal colonization is an important step in the characterization of the outbreak of clinical cryptococcosis on Vancouver Island.

Keywords Canada, cat, Cryptococcus gattii, dog

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 subtropics, particularly in association with eucalyptus trees [4–6]. 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. This area encompasses a portion of southeastern Vancouver Island, some small islands in the Strait of Georgia and a narrow strip of the adjacent mainland (Fig. 1). The CDF region is characterized by its wet, mild winters and dry, warm summers [9]. Since 2001, C. gattii has been repeatedly and consistently isolated from soil, air and vegetation within the CDF zone [10]. 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 [6,11–13]. In Australia, it has been reported that dogs, cats [14] and koalas [15] can carry C. neoformans in their upper respiratory tract asymptptomatically, suggesting that nasal colonization may be much more common than clinical disease. The following study was conducted to identify the prevalence 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.

Materials and methods

Study population

Five veterinary clinics in four cities (Fig. 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 6 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 3 weeks from June to December 2003.

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.

Animal sampling

Superficial nasal cultures were collected from unseated animals. In dogs a single Starplex StarSwab II (Starplex Scientific, Etobicoke, ON) moistened in transport media was inserted 0.5–2.0 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, Darmstadt, Germany).

Fig. 1 Location of sampling clinics (clear circles) and distribution of the Coastal Douglas Fir Biogeoclimatic zone on Vancouver Island, BC, Canada.
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 1 ml blood was collected from each animal participating in the study, allowed to clot for 15–30 min at room temperature and centrifuged to separate the serum.

Culture

Culture swabs were plated onto bird seed agar and incubated at 30°C. Plates were examined at 48 h, then daily for 7 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).

Antigen test

Samples were treated with pronase [16] 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, Cincinnati, OH). The CALAS test cannot identify the organism beyond the (CALAS); Meridian Bioscience, Cincinnati, OH). The CALAS test cannot identify the organism beyond the level of the genus. Animals with a titer ≥1:2 were considered positively infected with Cryptococcus spp. [17].

Statistical analyses

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 kg.

Descriptive and comparative statistics were computed using SPSS 12.0 (SPSS, 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-test (MWU) was used to compare means between positive and negative animals. The relative proportions of infected species were calculated using odds ratios and 95% confidence intervals. The prevalence of positive animals by city was evaluated using a chi-squared test. Kappa statistics were calculated to determine the agreement between the two nasal swabs and between the antigen test and nasal culture.

Results

Of serum samples collected from 84 cats, six (7.1%) and had an antigen titer ≥1:2 (Table 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, seven (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, range 0.7–18, years) and was not significantly different from cats who were not (Table 1). Positive animals and odds ratios for cats relative to dogs tested on Vancouver Island, BC, Canada

<table>
<thead>
<tr>
<th></th>
<th>Feline</th>
<th>Canine</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any test</td>
<td>7/95</td>
<td>5/283</td>
<td>4.42</td>
<td>1.37, 14.28</td>
</tr>
<tr>
<td>CALAS</td>
<td>6/84</td>
<td>2/266</td>
<td>10.15</td>
<td>2.01, 51.31</td>
</tr>
<tr>
<td>Total culture</td>
<td>4/94</td>
<td>3/280</td>
<td>4.10</td>
<td>0.90, 18.68</td>
</tr>
<tr>
<td>Superficial</td>
<td>3/94</td>
<td>3/280</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Deep</td>
<td>2/13</td>
<td>0/34</td>
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CALAS: Cryptococcal Antigen Latex Agglutination System.
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 (range 0.5–18) years, which was not statistically different from the negative cats (MWU, $P = 0.44$).

The odds of being positive on one or more test were not statistically different for male relative to female cats (OR 1.27, 95%CI 0.27, 6.03). The odds of a positive test result was 3.33 times (95%CI 0.320, 34.71) 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 3.99 times (95%CI 0.44, 36.01) 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 times (95%CI 0.35, 28.72) 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 kg (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 were 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 times (95%CI 0.90, 18.68) 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%) animals had a positive nasal culture but negative antigen test and five 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.

**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 had 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 [18–20]. 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 owner-perceived illness. Further investigation into the relationship between the incidence of asymptomatic infection and clinical disease is warranted. Animals with sub-clinical infection are being followed-up to determine whether or not they clear the organism or progress to disease.

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 [15], 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 [21]. 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 [14] and koalas [15,22]. 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 [23,24], 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 than did 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 [20] and clinical disease has been reported with equal or greater frequency in cats than in dogs [19]. Likewise, on Vancouver Island, the reported number of clinical cases in cats outweighs that in dogs [8].

Previously published studies on risk factors for animal cryptococcosis focus only on clinical cases. Cryptococcal disease has been reportedly more common in young large dogs [13,25] and in male cats [11,26,27], 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 [13,28]. While C. gattii has only been identified within the CDF zone on Vancouver Island [10], 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 may 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 [22]. 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.

References


