

# Ferrets as Sentinels of the Presence of Pathogenic *Cryptococcus* Species in the Mediterranean Environment

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**Abstract** *Cryptococcus gattii* is a pathogenic environmental yeast that is considered to be emerging in different areas of the world including the Mediterranean Basin. Exposure to infection might be more likely in animals than in human beings, given their closer relationship with the natural habitat of the yeast, vegetation and soil. Thus, animals, and especially pets, can act as indicators of the presence of this yeast in a determined area. Domestic ferrets (*Mustela putorius furo*) have become common pets in the past 10–20 years. Their natural behavior of sniffing around and going inside narrow spaces makes them prone to contact with decaying organic matter and soil, the substrate for *Cryptococcus* species. This study describes two cases of cryptococcosis in ferrets in the Iberian Peninsula and Balearic Islands and documents a relationship of ferret

cryptococcosis with environmental isolates in the same locations. Here, we emphasize the importance of how an adequate identification and environmental search of the yeast leads to a better understanding of the epidemiology of cryptococcosis and suggests ferrets may act as sentinels for this fungal disease.

**Keywords** *Cryptococcus* spp · Ferret · Mediterranean · Sentinel

## Introduction

Cryptococcosis is a disease caused by the basidiomycetous yeasts *Cryptococcus neoformans* and *Cryptococcus*

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*gattii*. The latter can be found in different species of trees [1, 2] with a distribution traditionally restricted to tropical and subtropical areas, although it has also emerged as a significant pathogen in temperate areas [2–4]. *C. neoformans* is a more ubiquitous species that can be found in trees as well as in bird feces. It usually causes disease in immunocompromised patients, such as those with underlying disease or having undergone organ transplantation [5].

Animals and human beings become infected by inhaling basidiospores or desiccated yeast cells [6], and they can remain asymptomatic, clear the infection or develop a serious disease [7].

Diagnosis can be achieved by examining cytological preparations or biopsy/necropsy tissues, or by testing for cryptococcal antigen in body fluids [8]. These tests do not allow differentiation to the species level, so further tests such as culture, specific phenotypic tests and genotypic characterization need to be performed [5, 9].

*C. gattii* and *C. neoformans* have traditionally been classified in varieties and serotypes [5]. Currently, species belonging to the *C. neoformans/C. gattii* complex are also classified according to their genotypes obtained by methods such as *PLB1* and *URA5* gene restriction fragment length polymorphism, multi-locus sequence typing, multi-locus microsatellite typing and amplified length fragment polymorphism (AFLP) [3, 5, 10–12]. This classification is summarized in Table 1.

The Mediterranean Basin is one of the areas where *C. gattii* is emerging. Extensive genetic analysis of human, animal and environmental isolates showed that the dominant genotype is AFLP4/VGI and that

isolates within this genotype are genetically homogeneous based on multi-locus sequence typing [4, 11].

Ferrets are carnivorous mammals that have recently become a common pet in Mediterranean countries. They live close to the ground and spend most of their active time sniffing surfaces to investigate their environment [13]. This makes them likely to get in close contact with decaying organic matter, which builds up around the roots and hollows of trees, the kind of substrate where *Cryptococcus* cells can be found [2, 5].

The current study presents two new cases and a review of the occurrence of cryptococcosis in ferrets in the Iberian Peninsula and Balearic Islands and assesses the relationship between ferrets, clinical isolates and the presence of *Cryptococcus* in the surrounding environment. These results remark the important role that this species can play to prevent human cases acting as sentinels of the presence of the yeast in the environment, as well as for the study of the environmental distribution of pathogenic *Cryptococcus* species.

## Case Report

### Case 1

A 13-month-old spayed female ferret from Palma de Mallorca (Balearic Islands, Spain) was referred to Clínica La Vileta due to a chronic sneezing that was not responsive to antibiotic treatment. Chest radiographs showed a bronchial pattern, and a course of enrofloxacin (10 mg/kg/po/bid) and meloxicam (0.2 mg/kg/po/sid)

**Table 1** Current classification of *C. gattii* and *C. neoformans*

	Species	Varieties	Serotype	Genotype	
				MLST	AFLP
<i>C. neoformans</i>	<i>C.n. var grubii</i>	Hybrid	A	VNI	AFLP1
			AD	VNII	AFLP1A
			D	VNIII	AFLP3
	Hybrid	–	AB	VNIV	AFLP2
			BD	VNI/VGI	AFLP9
<i>C. gattii</i>	–	–	B	VNIII/VGI	AFLP8
			C	VGI	AFLP4
				VGII	AFLP6
				VGIV	AFLP10
				VGIII	AFLP5
	VGIV	AFLP7			

MLST multi-locus sequence typing, AFLP amplified length fragment polymorphism



**Fig. 1** Radiograph of ferret number one showing an osteolytic lesion of the left zygomatic arch (white arrow)

was initiated. Twenty-eight days later, a mass on the left nasal area was found which corresponded radiographically to an osteolytic lesion affecting the nose and left zygomatic arch (Fig. 1). There was also left prescapular lymphadenomegaly. Bloodwork is summarized in Table 2. Due to failure of a previous antibiotic treatment and clinical presentation, a fungal infection was suspected as the cause of disease and therefore a 5-week course of itraconazole (5 mg/kg/po/sid) was initiated. Aspirates of the nasal mass were analyzed cytopathologically and mycologically. The cytological evaluation was non-diagnostic, but the fungal culture was positive and subsequent phenotypic tests revealed *C. neoformans* as the causative agent. The isolate was subsequently molecularly characterized using AFLP [10] as *C. neoformans* var. *grubii* genotype AFLP1/VNI. The cryptococcal isolate was deposited in the yeast collection of the Miguel Hernández University (Alicante, Spain) with accession number CCA393. Serologic testing for Aleutian disease virus (ferret parvovirus) was also found positive with an IgM titre of 1/40.

The patient's condition worsened, and a course of prednisolone (2 mg/kg/po/sid) was initiated; this, however, did not improve the condition of the ferret, and it was humanely euthanized 4 months after presentation. Postmortem studies were not performed.

#### Case 2

A 1-year-old neutered male ferret from Vila Nova de Milfontes (Portugal) was presented with cough of

2-month duration. Bloodwork results are shown in Table 2. A mass on the dorsal aspect of the caudal lobe of the right lung was observed on chest radiographs (Fig. 2). Cytological analysis of this mass obtained by fine-needle aspiration revealed large colonies of yeast like cells with a thick clear halo that did not pick up Indian ink morphologically suggestive of *Cryptococcus* spp. A 6-week course of itraconazole (10 mg/kg/po/sid) was initiated and the cough resolved during the fourth week of treatment. At that time, a second sample was taken by fine-needle aspiration from the same thoracic mass. The culture in brain–heart infusion broth and on Sabouraud dextrose agar yielded yeast colonies that were identified as *C. gattii* and molecularly characterized by AFLP fingerprinting as genotype AFLP6/VGII. The cryptococcal isolate was deposited in the yeast collection of the CBS-KNAW Fungal Biodiversity Centre with accession number CBS12659. Control chest radiographs were taken 2 months later and did not show any masses. Cryptococcal blood antigen levels were undetectable. One year after the initial presentation, this ferret was found dead without any prior respiratory symptoms. Postmortem studies were not performed.

**Case 3** A 17-month-old neutered male ferret showing mandibular lymphadenopathy and blindness was diagnosed of cryptococcosis by *C. gattii* in Barcelona, Spain [15]. The yeast was molecularly characterized as genotype VGI/AFLP4 and deposited in the yeast collection of the CBS-KNAW Fungal Biodiversity Centre with accession number CBS11807.

#### Detection of Asymptomatic Carriers

The owner and another ferret from the same household of case 2 were tested for asymptomatic carriage of *Cryptococcus* species in the nasal mucosa with a negative result. A similar investigation lead to the detection of asymptomatic carriers of *C. gattii* in the household of case 3 [15].

#### Environmental Search

Appropriate sampling of trees (olive—*Olea europaea*—and carob—*Ceratonia siliqua*—trees) and associated soils was made in different areas of

**Table 2** Blood test results of ferrets 1, 2 and 3

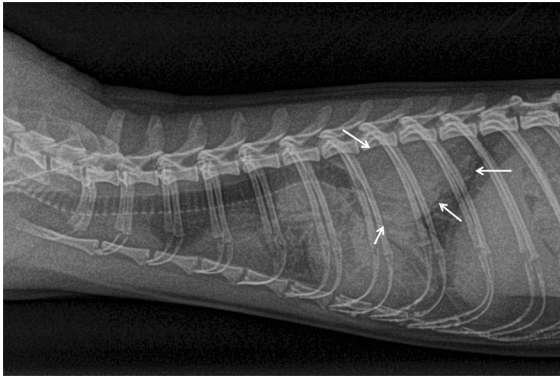
Date	Ferret 1		Ferret 2	Ferret 3		Reference values [25]
	5/09/11	18/11/11		25/06/08	16/09/08	
<i>Hematology</i>						
Packed cell volume (%)	<b>15.42</b>	<b>20.97</b>	<b>34.7</b>	<b>56.4</b>	<b>49.4</b>	36–48
Hemoglobin (g/dl)	<b>5.4</b>	<b>7.7</b>	12.4	15.5	15.8	12.2–16.5
Red blood cells ( $\times 10^6/\mu\text{l}$ )	<b>3.71</b>	<b>4.8</b>	<b>6.92</b>	9.35	9.42	7.01–9.65
White blood cells ( $\times 10^3/\mu\text{l}$ )	<b>4</b>	<b>4.22</b>	6.1	6,900	6,400	4.3–10.7
Neutrophils (%)	43.3 <sup>a</sup>	52.4 <sup>a</sup>	<b>78.3</b>	<b>82</b>	<b>57</b>	18–47
Lymphocytes (%)	50.4	45.1	<b>16.5</b>	<b>17</b>	<b>33</b>	41–73
Monocytes (%)	<b>6.3</b>	2.4	1	1	<b>5</b>	0–4
Eosinophils (%)			2.5	0	<b>5</b>	0–4
Basophils (%)			1.7	0	0	0–2
Bands (cells/ $\mu\text{l}$ )	–	–	–	0	0	–
Platelets ( $10^3/\mu\text{l}$ )	<b>108</b>	<b>70</b>	<b>831</b>	<b>501</b>	<b>690</b>	200–459
Mean corpuscular volume (fL)	<b>42</b>	<b>44</b>	50	<b>60.3</b>	52.4	50–54
Mean corpuscular hemoglobin (pg)	<b>14.5</b>	16.1	17.9	16.6	16.8	15–18
Mean corpuscular hemoglobin concentration (g/dl)	34.8	<b>36.8</b>	35.7	<b>27.5</b>	32	32–35
<i>Plasma biochemistry</i>						
Alanine aminotransferase (U/l)	<b>31</b>	<b>51</b>	<b>53</b>		<b>40</b>	65–128
Alkaline phosphatase (U/l)	<b>18</b>	<b>8</b>	26			25–60
Amylase (U/l)	<b>10</b>	<b>12</b>				26–36
Aspartate aminotransferase (U/l)			<b>64</b>			70–100
Bilirubin, total (mg/dl)	0.2	0.3				0.2–0.5
Blood urea nitrogen (mg/dl)	27	<b>33</b>			<b>74</b>	18–32
Calcium (mg/dl)	8.9	8.2			7.68	8.1–9.5
Creatinine (mg/dl)	<b>&lt;0.2</b>	<b>&lt;0.2</b>			<b>0.78</b>	0.2–0.5
Creatine phosphokinase (U/l)					<b>188</b>	55–93
Glucose (mg/dl)	<b>134</b>	<b>122</b>				80–117
Phosphorus (mg/dl)	<b>7.8</b>	5.6			5.46	5.1–6.5
Potassium (mmol/l)	5.4	5.4			5	4.5–6.1
Sodium (mmol/l)	<b>137</b>	<b>135</b>			<b>140</b>	142–148
<i>Plasma protein electrophoresis</i>						
Total protein (g/dl)	<b>11.5</b>	<b>11.3</b>		<b>8.8</b>	<b>6.5</b>	4.5–6.2
Albumin (g/dl)	<b>1.81</b>	<b>1.95</b>			3.18	2.5–3.31
Alpha-1 globulin (g/dl)	0.48	<b>0.7</b>			<b>0.8</b>	0.33–0.56
Alpha-2 globulin (g/dl)	<b>0.68</b>	<b>1.03</b>			0.54	0.36–0.6
Beta globulin (g/dl)	<b>2.05</b>	<b>3.18</b>			<b>2.04</b>	0.83–1.2
Gamma globulin (g/dl)	<b>6.49</b>	<b>4.44</b>			0.57	0.31–0.81
Albumin/globulin ratio	<b>0.19</b>	<b>0.21</b>			<b>0.96</b>	1.05–1.33

Abnormal results are put in bold

<sup>a</sup> Expressed as total granulocyte count

Mallorca Island (Spain). In Vila Nova de Milfontes (Portugal), samples of soil and swabs from four false pepper trees (*Schinus molle*) around the house of the

infected animal were obtained. Samples were processed for the search of pathogenic *Cryptococcus* species [4].



**Fig. 2** Thoracic radiograph (laterolateral view) of ferret number two showing a pulmonary mass (white arrows)

### Identification and Typing of *Cryptococcus* Species Isolates

Colonies suggestive of *Cryptococcus* species grown in cultures of clinical and environmental samples were isolated and phenotypically identified [4] as members of the *C. neoformans*/*C. gattii* species complex; these colonies were subsequently molecularly characterized [10].

### Discussion

A recently published review of cryptococcosis in ferrets gathered a total of 16 cases from 1954 to 2011 [14]. Of these, two cases came from Europe, one from the United Kingdom and the other from Spain [15], the latter corresponding to case number three in the current study. No other cases of cryptococcosis in ferrets have been reported since then.

The two newly described cases of cryptococcosis in ferrets in this study corresponded to infections caused by *C. neoformans* variety *grubii* genotype AFLP1/VNI (case 1) and by *C. gattii* genotype AFLP6/VGII (case 2). Environmental sampling in the vicinity of the house where the ferret from case 1 resided yielded five isolates of the same species, variety and genotype, namely *C. neoformans* var. *grubii* genotype AFLP1/VNI (Table 3). The molecular characterization of these isolates and their genetic profiles evidenced a very close relationship between them suggesting the same origin and the acquisition of the disease from natural sources. In case 2, all environmental samples obtained from the surroundings of the house, as well as nasal swabs from the owner and a companion ferret, were negative.

**Table 3** Summary of the strains isolated from clinical and environmental samples, ID and genotype

Strain N°	Origin		ID	Genotype	Reference
CCA393	Clinical case 1	Mallorca (Spain)	<i>C. neoformans</i> var. <i>grubii</i>	AFLP1/VNI	This article
CCA351	Carob tree				
CCA352	Carob tree				
CCA353	Carob tree				
CCA355	Olive tree				
CCA356	Olive tree				
CBS12659 (CCA358)	Clinical case 2	Viana de Mil fontes (Portugal)	<i>C. gattii</i>	AFLP6/VGII	This article
CBS11807	Clinical case 3	Gavà Barcelona (Spain)	<i>C. gattii</i>	AFLP4/VGI	Morera et al. [15]
CBS11808-11810	Ferrets asymptomatic carriers				
CBS11811-11812	Human asymptomatic carriers				
CBS11813-11817	Carob tree				
CBS11819 CBS11826 CBS11828					
CBS11749-11750					
CBS11751	Pine tree				

Cryptococcosis in ferrets has been reported in areas where the presence of *C. gattii* has been extensively documented, such as Australia and Vancouver Island (Canada) [16, 17]. Reports from other geographical areas are scarce [14]. The low number of cases in ferrets may be due to a real low incidence but also to the fact that ferrets became popular pets quite recently. This social change could increase significantly the chance for the detection of diseases affecting this species. Therefore, it is difficult to infer whether these cases of cryptococcosis in ferrets correspond to a real epidemiological change in the global distribution of *Cryptococcus* in the environment.

The detection of three ferret cases infected with different genotypes of two different pathogenic species of *Cryptococcus* is meaningful and suggests the presence of a diversity of pathogenic *Cryptococcus* species in the Mediterranean environment. Case 2 is considered particularly relevant since it describes the first local animal infection caused by an isolate belonging to the hypervirulent *C. gattii* AFLP6/VGII genotype.

Cryptococcosis in case 1 was caused by *C. neoformans* genotype AFLP1/VNI (serotype A), known as *C. neoformans* var. *grubii*, which is the most common genotype found among immunocompromised human patients [18], although it appears to be a primary pathogen in dogs, cats and ferrets [19]. In this case, infection involved an Aleutian disease seropositive ferret: Aleutian disease virus can cause immunosuppression [20], which might have contributed to a fatal outcome in case 1. Treatment with itraconazole, which is routinely used to treat cryptococcosis in cats and dogs [8], has been successful in clearing this infection in ferrets, although the doses reported in the literature have been significantly higher (15–20 mg/kg/po/sid [16, 19], 10 mg/kg/po/bid [21]) than the ones used in this ferret.

Case 2 was found to be caused by *C. gattii* genotype AFLP6/VGII, the hypervirulent genotype that has been involved in various outbreaks across the globe [3, 11]. In this case, the environmental source of the isolated yeast was not found. A human case of cryptococcosis by this genotype has also been recently described in Portugal [22]. The previous detection of some *C. gattii* isolates in environmental samples in the same region [23] suggests a permanent source of this genotype in this country.

An environmental survey performed in Gavà (Barcelona, Spain) in relation to case 3 led to the detection

of *C. gattii* in carob trees that belonged to the same strain as the ferret isolate [4].

The absence of a postmortem examination for cases 1 and 2 does not allow to clarify the cause of death and to confirm that fungal infection was completely cleared in case 2.

Information regarding hematological abnormalities in ferrets with cryptococcosis is scarce and shows inconsistent results [14]. The data collected from the three cases in the current study (Table 2) only share abnormalities attributable to chronic disease (anemia, neutrophilia, lymphopenia). Case 1 had mild leukopenia and a marked increase in gamma globulins, which can be seen in Aleutian disease and other infectious diseases of ferrets such as systemic coronavirus infection [24]. Case 3 additionally had alterations of some biochemical parameters, probably due to brain and muscle damage and increased protein intake.

Diagnosing cryptococcosis from the practitioner standpoint be achieved either by identifying the yeast in cytological specimens or biopsy/necropsy tissues, or by detecting cryptococcal antigen in body fluids, although none of these tests is valid to determine the species or genotype involved. An adequate investigation of the genotype helps determining the virulence of the yeast and the potential danger for people in contact with the infected animal.

The ferret's natural behavior and its recent incorporation as a pet may make it a valuable sentinel species for *Cryptococcus* infection. The occurrence of at least three cases in Spain and Portugal during 4 years would support this statement. As dedicated owners bring their ill ferrets to the veterinarian's practice, the veterinarian should become the frontline to detect this pathogenic yeast.

If pathogenic species such as *C. gattii* are detected, the owners should be adequately informed to undertake tests to detect a potential asymptomatic carrier status.

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