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CRYPTOCOCCOSIS IN GILBERT'S AND LONG-NOSED POTOROO

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Abstract: Two cases of fatal cryptococcosis are described, one of *Cryptococcus neoformans* infection in a Gilbert's potoroo (*Potorous gilbertii*) and one of *Cryptococcus gattii* infection in a long-nosed potoroo (*Potorous tridactylus*). The diagnoses were confirmed by culture and specific immunohistochemistry, respectively. The long-nosed potoroo tested positive using the latex cryptococcal antigen test (LCAT), whereas the Gilbert's potoroo had a negative LCAT result despite having advanced disease of some duration. In both cases, the clinical presentation was a progressive neurologic disease associated with a central nervous system infection. Pulmonary infection was also observed in the long-nosed potoroo. Specific treatment with antifungal agents was unsuccessful in the long-nosed potoroo.

Key words: Potoroo, *Cryptococcus neoformans*, *Cryptococcus gattii*, itraconazole, marsupial.

INTRODUCTION

With the exception of the koala,[15] cryptococcosis is rarely reported in marsupials, and no cases have been described in potoroids. Cryptococcosis in mammals is caused by two species: *Cryptococcus neoformans* (var. *grubii* and var. *neoformans*) and *Cryptococcus gattii* (formerly *C. neoformans* var. *gattii* or *Cryptococcus bacillisporus*).[13] Infection is acquired from the environment, primarily via inhalation.

In humans, disease caused by *C. neoformans* is classically associated with immunocompromised individuals, whereas *C. gattii* usually causes disease in immunocompetent hosts. In other mammals, however, both *C. neoformans* and *C. gattii* can behave as primary pathogens of immunocompetent hosts.[14,15] Common presentations in mammals include rhinosinusitis, pneumonia, and disseminated disease, including meningitis.[14]

Potoroos are nocturnal marsupial rat kangaroos belonging to the family *Potoroidae*. The Gilbert's potoroo is Australia's most critically endangered mammal.[4] Its estimated population size of 30 individuals is found only within the Two Peoples' Bay Nature Reserve, 30 km east of Albany in Western Australia.[5] The mainland subspecies of the long-nosed potoroo, occurring in southeastern Australia, is classified as ''vulnerable'' in status.[4] Perth Zoo established a long-nosed potoroo colony in 2000 in order to refine dietary and husbandry requirements and to develop artificial insemination procedures for the closely related Gilbert's potoroo.[11]

This case report describes two cases of cryptococcosis, a case of *C. neoformans* infection in a Gilbert's potoroo and a case of *C. gattii* in a long-nosed potoroo.

CASE REPORTS

Case 1

A male Gilbert's potoroo pouch young was brought from the wild with its mother into the captive breeding colony housed at Two Peoples' Bay Nature Reserve in January 1995. In March 1999, it had a brief sneezing and coughing episode and developed a hunched appearance. On examination, it was trembling and appeared painful on palpation of its lower thoracic/lumbar spine. A tentative diagnosis of spinal muscle injury was made, but no treatment was administered. Respiratory signs abated, but the animal's appetite diminished and its body weight dropped from 1,096 g to 947 g (14% loss) in 2 wk.

By February 2000, the animal's body weight had declined to 863 g, a 21% weight loss from its initial

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weight at presentation; the animal's appetite had diminished, and another episode of apparent discomfort with a hunched appearance was seen. Hematologic and plasma biochemical testing revealed leukopenia and the presence of intraerythrocytic *Theileria*-like organisms. Such organisms had been seen in the blood of all of the potoroos in the captive colony and had not been associated with clinical signs (Vaughan, unpubl. data). No treatment was given and the animal improved over a week but was seen on one occasion to be circling in its pen.

The animal's weight continued to decline. In August 2000, at 748 g, 32% below its initial weight, the potoroo appeared sluggish and was observed stumbling. It appeared pale, was estimated to be 5% dehydrated, and was hypothermic (with a rectal temperature of 32.8°C). It was treated with subcutaneous 2.5% glucose in 0.9% saline (5% glucose and 0.9% sodium chloride solution; Baxter Healthcare, Old Toongabbe, New South Wales 2146, Australia) over 3 days but further deteriorated and was not able to use its forelimbs and was seen to intermittently circle and stumble to the left. It had no withdrawal reflex in the left foreleg and marked atrophy of the left shoulder muscles.

Treatment began with 12.5 mg of amoxicillin/clavulanic acid (Clavamox®, Pfizer Animal Health, West Ryde, New South Wales 2114, Australia), administered subcutaneously (s.c.) daily for 3 days, and 1 mg s.c. norandrosterone laurate (Laurabolin®, Intervet, Victoria 3550, Australia). Subcutaneous Vitamin B and E and Hartmann's solution® (Baxter Healthcare) were also administered.

Treatment was continued for 5 days with no clinical improvment. Several attempts at venipuncture failed, so the potoroo was anesthetized with isoflurane for blood collection and whole-body radiographs. The only radiographic abnormality noted was mild bilateral lucency of the nasal cavity. Hematology and plasma biochemical analyses were normal, apart from increased creatine kinase activity (4991 U/L), which was attributed to prolonged recumbency. Serum agglutination tests for Toxoplasma immunoglobulin G (IgG) and IgM antibodies were negative. Urine dipstick analysis showed 2+ glucose and 1+ protein.

The potoroo recovered from anesthesia to the point of ambulation but was later found comatose and twitching. Horizontal nystagmus was noted. Diazepam (0.1 mg, administered intramuscularly [i.m.]) (Pamlin®, Parnell Laboratories, Mascot, New South Wales 2015, Australia) initially seemed to calm the potoroo, but the convulsions continued. Further diazepam (0.2 mg i.m.) and dexamethasone (0.1 mg i.m.) (dexamethasone sodium phosphate injection®, Mayne Pharma, Parkville, Victoria 3052, Australia) were given. At this time, marked anisocoria and lateral nystagmus were observed. The potoroo settled but began convulsing again, and for the next 12 hr the patient was treated regularly with diazepam to control convulsions. Finally, it was anesthetized again, and blood was collected before the animal was euthanized with intracardiac pentobarbitone. The blood tested negative in the latex cryptococcal antigen test (LCAT)[12] using a CALAS kit incorporating a pronase pretreatment step (Meridian Bioscience, Cincinnati, Ohio 45244, USA).

Necropsy revealed extensive fat reserves. Stomach contents were stained with blood, and there was diffuse bleeding from the cardia region. There were multifocal widespread irregular areas of congestion in the liver, within which there were focal yellow areas up to 3 mm in diameter. In the subcutaneous tissues of the hind legs there was patchy congestion and hemorrhage, particularly in the muscles around the sciatic nerve. No gross lesions were noted in the central nervous system (CNS).

Histopathology revealed severe multifocal granulomatous meningoencephalomyelitis. Within vacuoles in macrophages, there were intracellular yeast-like bodies with peripheral spaces. There was considerable variation in the size of these bodies, with larger bodies of up to 30 μm often markedly distorted (Fig. 1). No obvious budding was identified. Lesions were widespread in the meninges of the cerebral cortex and in the subependymal areas of the lateral ventricles. Particularly prominent inflammatory foci were seen in the roof of the fourth ventricle and around the root of one vestibular nerve. In the spinal cord at the level of brachial plexus, a similar lesion obliterated most of the gray matter. In the white matter of the cord in this region there was a patchy vacuolar change with occasional swollen axons. In the cerebellum there was acute necrosis of numerous Purkinje cells. There were also moderate acute multifocal hepatic necrosis, superficially localized moderate gastric mucosal necrosis, diffuse acute mild renal tubular necrosis, mild multifocal membranous glomerulonephritis, and mild multifocal acute rhinitis. A specific immunohistochemical stain for *C. neoformans*[7] stained the organisms seen in hematoxylin and eosin (H&E) sections.

Case 2

In November 2004, a 4.5-yr-old female long-nosed potoroo from the Perth Zoo colony presented with loss of 80 g (27% of its body weight) over a

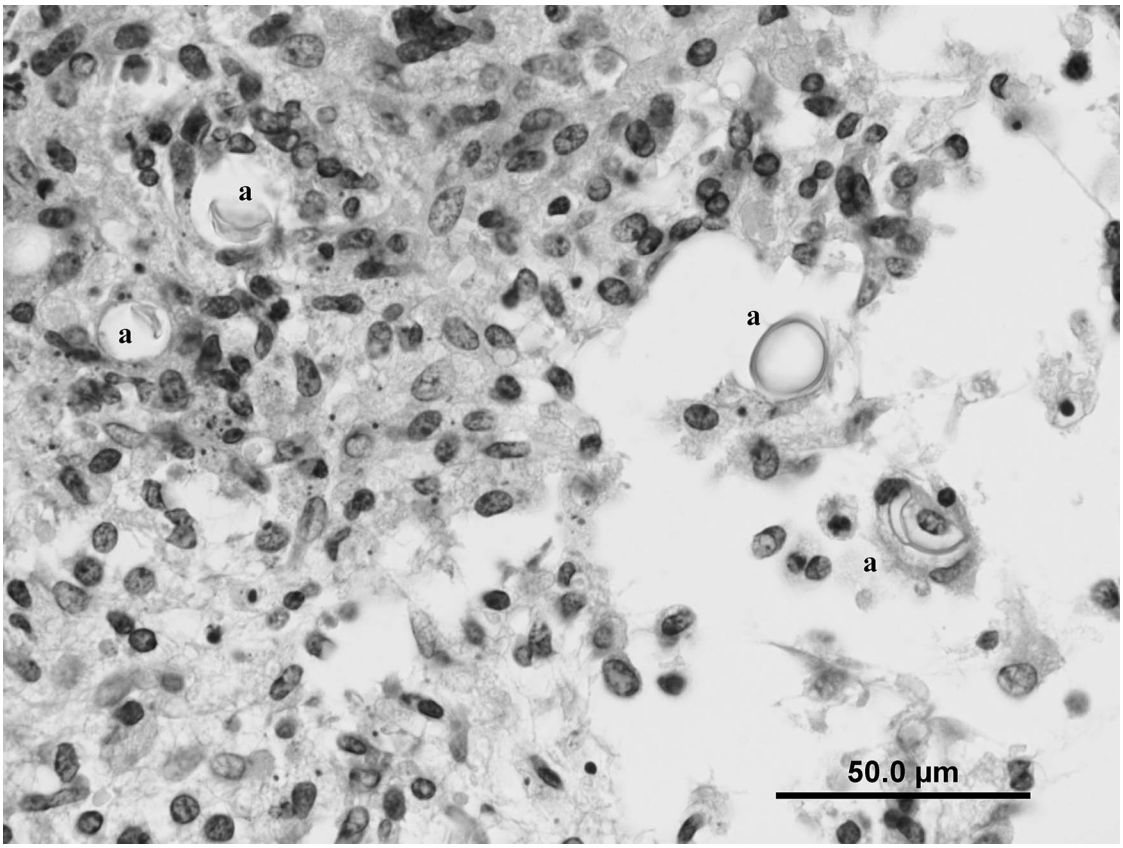

Figure 1. *Cryptococcus neoformans* in a Gilbert's potoroo. Granulomatous inflammatory lesion in the spinal cord with large distorted yeast forms (a). Hematoxylin and eosin (H&E), ×400.

6-wk period. This potoroo had a pouch young and a young at foot, both of which were removed to minimize further nutritional strain from lactation and to rule out competition for food with the young at foot.

In addition to weight loss, early presenting signs included hind limb weakness, gluteal muscle atrophy, and a rough hair coat. Physical examination under isoflurane anesthesia, including dental examination and whole-body radiographs, did not detect further abnormalities. Hematology and serum biochemistry were normal, except for a mild hyperbilirubinemia. *Toxoplasma* serum agglutination testing for IgG and IGM proved negative, and no evidence of parasitism was detected on fecal flotation. There was little clinical response to empiric therapy with combined amoxycillin–clavulanic acid, 12.5 mg, administered s.c. (Clavamox®, Pfizer Animal Health), and 25 ml of s.c. fluids (Hartmanns compound sodium lactate®, Baxter Healthcare), both of which were administered daily for 5 days.

The potoroo's condition deteriorated over the subsequent 5 days, and it was again anesthetized and given intravenous (i.v.) fluids and parenteral Vitamin B and Vitamin E. Repeat hematology and serum biochemistry testing revealed no significant findings. Therapy under general anesthesia was repeated the following day, and antibiotic therapy was changed to enrofloxacin (5 mg s.c., s.i.d; Baytril®, Bayer Animal Health Pty Ltd., Pymble, New South Wales 2079, Australia). Meloxicam (0.2 mg s.c., s.i.d; Metacam®, Boehringer-Ingelheim, North Ryde, New South Wales 2113, Australia) and norandrostenolone laurate (1 mg s.c.; Laurabolin®, Intervet, Victoria 3550, Australia) were also administered and an indwelling i.v. catheter placed.

Seven days following initial anesthesia, a slight and progressive head tilt to the right, circling to the right, and prolonged recumbency became apparent. Bilateral exopthalmos, visual deficits, and progressive ataxia ensued. The pupils were fixed and dilated, and upon ophthalmoscopic examination, retinal hemorrhage and optic neuritis were evident. Blood was taken for LCAT[12] using a CALAS kit

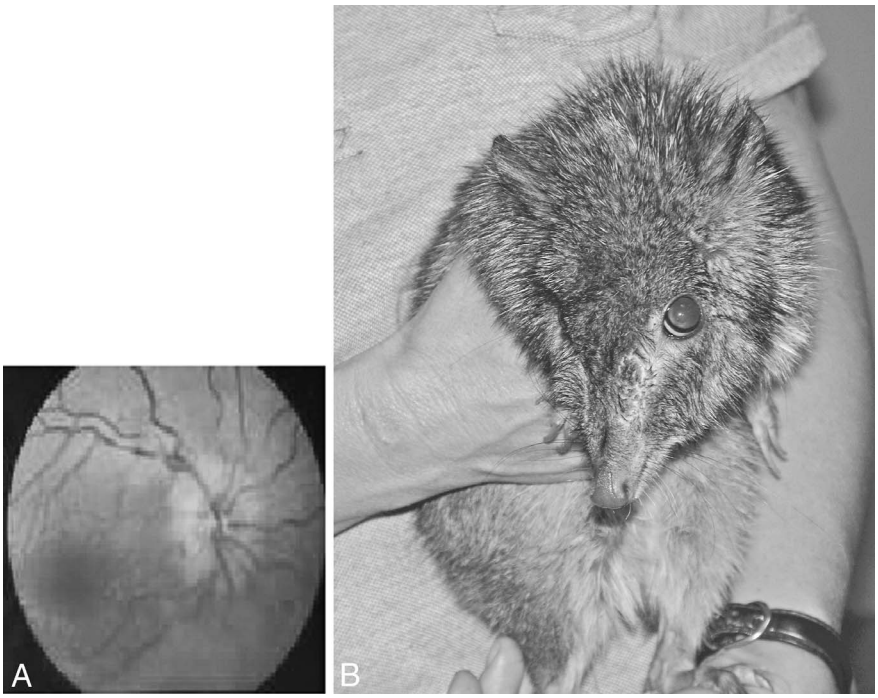

Figure 2. **A.** Retinal hemorrhage and papilloedema was seen associated with optic neuritis. **B.** Exopthalmos and fixed dilated pupils were prominent.

incorporating pronase (Meridian Bioscience). Presumptive treatment for cryptococcosis was initiated with itraconazole (20 mg/kg s.i.d, by mouth [p.o.]; Sporanox®, oral liquid Janssen–Cilag, North Ryde, New South Wales 2113, Australia). The ocular lesions were treated with 0.5% dexamethasone topical drops, administered four times a day (q.i.d.; Maxidex®, Alcon Laboratories, French's Forest, New South Wales 2086, Australia). Enrofloxacin was continued prophylactically in case of secondary bacterial infection. The LCAT test result was positive, with a titer of 1:32. This titer and clinical signs were consistent with a diagnosis of neural and ocular cryptococcosis.[12]

Inappetence continued, necessitating supplemental syringe feeding. Although the potoroo appeared brighter in demeanor following the start of antifungal treatment, circling to the right persisted and was exacerbated by stress. Ocular lesions showed no improvement. After 1 wk of treatment with itraconazole, antifungal therapy was changed to fluconazole (10 mg/kg p.o., two times a day [b.i.d.]; Diflucan®, Pfizer Animal Health). Additionally, 0.7 mg of amphotericin B (0.14 ml of the 5 mg/ml Fungizone® solution, Bristol–Myers Squibb, Noble Park, Victoria 3174, Australia) was administered subcutaneously twice weekly, diluted in 50 ml of 0.45% NaCl and 2.5% glucose (sodium chloride and glucose solution, Baxter Healthcare).[2] The amphotericin B infusion was administered under anesthesia to minimize stress to the animal. No cryptococcal organisms were cultured from nasal flushes, and repeat radiographs did not show any evidence of pulmonary involvement.

Ophthalmoscopic examination 25 days following the initial anesthesia revealed significant optic neuritis and absence of the pupillary light reflex in the right eye (Fig. 2). Similar but less severe changes were present in the left eye. These findings, combined with the potoroo's behavior, were consistent with partial blindness. The ocular dexamethasone drops were reduced to b.i.d. administration to minimize handling. Repeat monitoring of hematology and serum biochemistry indicated normal renal function, despite the potentially nephrotoxic effects of amphotericin B.

A repeat LCAT 40 days after the initial positive LCAT showed a greatly increased titer of 1:8,192. Consequently, a decision was made to increase doses of amphotericin B to 0.9 mg per treatment and to increase the fluconazole to 20 mg p.o., administered b.i.d.

One week later, polydipsia became evident. However, there were no other clinical signs or bio-

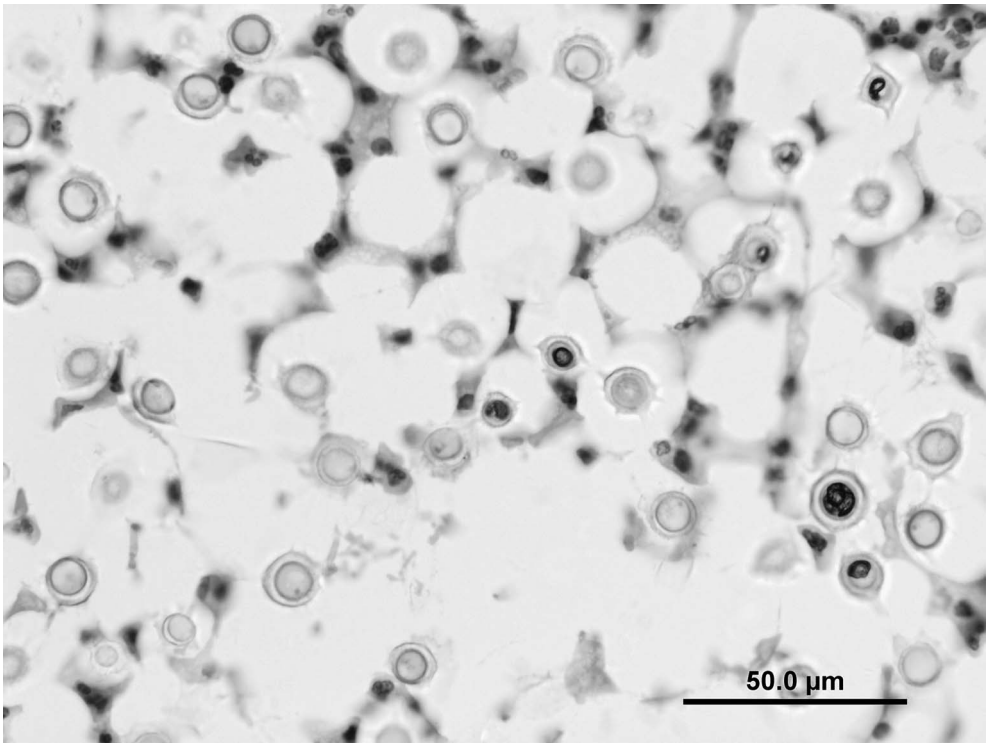

Figure 3. *Cryptococcus gattii* in a long-nosed potoroo. Typical soap-bubble lesion in olfactory lobe of the cerebral cortex. Hematoxylin and eosin (H&E), ×400.

chemical evidence of azotemia. Two weeks following implementation of the new dosage regimen, the animal became inappetant. Conjunctivitis developed in the left eye and was treated with chloramphenicol ointment, administered b.i.d. (Chloromycetin®, Pfizer Animal Health).

Given the lack of improvement in neurologic status and the onset of secondary problems, a decision was made to euthanize the potoroo. The animal was anesthetized and then euthanized with i.v. pentobarbitone (Lethabarb®, Virbac Australia, Peakhurst, New South Wales 2015, Australia).

Histopathology revealed disseminated meningoencephalitis, optic neuritis, granulomatous rhinitis, and pneumonia. Both the respiratory and nervous tissue lesions had a typical ''soap-bubble'' appearance, with narrow-necked budding yeast-like bodies (Fig. 3). *Cryptococcus gattii* was cultured from the cerebral tissue and from a sample of cerebrospinal fluid collected aseptically immediately after the animal's death.

DISCUSSION

Some common features are evident in these two cases, even though two different species of *Cryptococcus* were involved and although different species of potoroo were affected. Severe granulomatous meningoencephalitis was considered to be the cause of the clinical syndrome in both potoroos, with lesions in other organs considered secondary. Hind limb weakness, muscle atrophy, ataxia, and circling were consistent clinical findings. Visual deficits were evident in both potoroos, and marked retinal hemorrhage and optic neuritis were seen in the long-nosed potoroo.

The organisms identified as *C. gattii* in lesions in the long-nosed potoroo had a typical ''soap-bubble'' appearance, whereas the organisms identified as *C. neoformans* in the Gilbert's potoroo were fewer and were embedded in a granulomatous inflammatory reaction. The organisms in the latter case were variable in size and often displayed a distorted appearance. The distortion is likely an artifact of processing, but was consistent within a number of sections.

Clinical diagnosis of cryptococcosis was confirmed in the long-nosed potoroo by the positive LCAT test. Cryptococcosis was suspected because of the clinical experience gained from handling the case in the Gilbert's potoroo.

It is likely that in both cases the respiratory tract was the primary site of infection, following inhalation into the lung, with subsequent central nervous and hematogenous dissemination. Alternatively, the episode of respiratory disease in the Gilbert's potoroo could possibly have been caused by a primary cryptococcal rhinitis. However, the lack of lesions in the nasal cavity and the long period of time between this episode and the illness preceding death makes this possibility unlikely.

Cryptococcosis has rarely been reported in marsupials other than the koala.[1,8,10] Cases of cryptococcosis in quokkas (*Setonix brachyurus*), wallabies (species unspecified), and numbats (*Myrmecobius fasciatus*) were found in the archives of the Western Australian Department of Agriculture and Food Animal Health Laboratory, and cases of cryptococcosis in quokkas, a red-necked wallaby (*Macropus rufogriseus banksianus*), a dusky antechinus (*Antechinus swainsonii*), and a feathertail glider (*Acrobates pygmaeus*) were found in the files of the Registry of Wildlife Pathology at Taronga Park Zoo (Rose, pers. comm.). One case of cryptococcal granulomatous meningitis, optic neuritis, and multifocal granulomatous encephalitis in a long-nosed potoroo was also found in the Registry of Wildlife Pathology at Taronga Park Zoo. This indicates that cryptococcosis is not uncommon in marsupials. The low number of cases in potoroids could be a reflection of a small sample size, as there are few potoroos in captivity.

Although it is possible that both species of *Cryptococcus* can cause disease in immunocompetent potoroos, immune dysfunction is a possible predisposing condition in these cases. A study of mycobacterial disease in long-nosed potoroos indicates that susceptibility to this disease is related to immunologic status and/or regulation.[15] Studies of immunologic function in both the captive breeding and wild populations of Gilbert's potoroos are currently being undertaken to determine whether the described cases of cryptococcosis are an indication of immune dysfunction of the population.

The habitat of potoroos should also be considered a factor that could increase susceptibility to disease. Wild potoroos, being ground dwellers and feeding on underground fungus, primarily inhabit soil substrates, yet they will come into contact with moist leaf litter containing *Eucalyptus* sp. and bird guano. Captive Gilbert's potoroos housed within the Two Peoples' Bay captive breeding facility also exist primarily on a sand substrate. Sand changes in the proximal quarter of the enclosures occur quarterly, as this section of sand is not exposed to the rain and organic matter can build up in this area. Three quarters of the enclosure roof consists of wire mesh and is open to the elements, so passing birds could defecate through the wire. These factors may expose the potoroos to infectious propagules, increasing their potential for exposure and subsequent infection.

Treatment regimes are yet to be established for cryptococcosis in marsupial species. Amphotericin B (often combined with 5-flucytosine) is recognized as the gold standard in the treatment of cryptococcosis, owing to the weight of evidence-based medicine supporting its use and because it is fungicidal rather than fungistatic.[3] However, given its potential for reversible nephrotoxicity, it is usually reserved for life-threatening cases with CNS involvement and for cases that are unresponsive to azoles. The decision was made to initially commence treatment with itraconazole, an agent that could be given orally without recourse to general anesthesia. However, it soon became clear that itraconazole was having little effect in reversing neurologic signs, and therapy was therefore changed to include the combination of amphotericin B and fluconazole. Fluconazole achieves better CNS penetration than itraconazole, with generally fewer side effects.[3] Although the use of 5-flucytosine may have been helpful, it was not possible to obtain this agent in a timely fashion or in a suitable formulation.

Historically, amphotericin B has been administered i.v. to human, canine, and feline patients. However, newer subcutaneous infusion methods[2] not only slow absorption of the potentially nephrotoxic drug and promote diuresis to help minimize nephrotoxicity but also make administration possible under manual restraint. The authors have used this approach in a variety of zoo animals, including koalas and a cheetah. Although the potoroo still required light general anesthesia for administration of amphotericin B, its small size and lack of robust veins for venipuncture made the s.c. route a practical route of administration.

In cases involving optic neuritis, many authorities recommend concurrent administration of corticosteroids. However, owing to the underlying infectious etiology, steroids were initially withheld in favor of NSAID therapy. Corticosteroids were introduced following the second administration of amphotericin B, when neurologic signs became worse immediately following treatment. This phenomenon was thought to be related to the osmotic effects of polysaccharides released from the dead fungus and to the inflammatory response to the intracellular cryptococcal antigens of the host.[2] The decision was therefore made to premedicate with

dexamethasone (0.5 mg/kg) before subsequent amphotericin B infusions in order to minimize adverse neurologic sequelae related to this phenomenon.

Environmental colonization with *C. gattii* (molecular type VG1) has been associated with bark and plant debris from certain eucalyptus species, including *Eucalyptus camaldulensis* (river redgum), *Eucalpytus tereticornis* (forest redgum), *Eucalyptus grandis* (flooded gum), *Eucalyptus rudis*, and *Eucalyptus gomphocephala*.[13–15] One possible source of contamination of the potoroo enclosures at the Perth Zoo is the eucalyptus branches that were used as enclosure furniture. These branches were commonly recycled from the zoo's koala enclosure. This may be a significant factor, since koalas have been found to be capable of amplifying the number of cryptococci in their environment.[6,9,13] Cryptococcal culture of sand, branches, and leaves from the koala enclosure is being undertaken to validate this presumption.

CONCLUSIONS

Cryptococcosis represents a potential threat to the survival of both the critically endangered Gilbert's potoroo and the vulnerable long-nosed potoroo. In species with small population sizes, the effects of disease can be catastrophic. Over time, these small populations can also lose genetic variability as a result of genetic drift and/or inbreeding, which may in turn increase susceptibility to disease.[5] Cryptococcal screening via nasal swab, LCAT titers, and environmental sampling should be considered in captive breeding and translocation health screening protocols for potoroos.

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