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A Case of Pulmonary Cryptococcosis in a Free-living Toad (*Bufo bufo*)

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ABSTRACT: Pulmonary cryptococcosis was observed in a free-living adult female common toad (*Bufo bufo*) that was killed by a vehicle. Both lungs had various eosinophilic, monomorphic, and spherical to elliptical organisms identified as *Cryptoccocus* spp. The yeasts were demonstrated by Grocott's silver method and the periodic acid-Schiff reaction and the capsule was positive for mucin with a mucicarmine stain. The agent was confirmed by immunohistochemistry, using the monoclonal antibody anti-*Cryptococcus neoformans*, and by a polymerase chain reaction-based method using a *C. neoformans*-specific primer. This report, to the best of our knowledge, represents the first case of cryptococcosis in a common toad.

Key words: *Bufo bufo*, cryptococcosis, *Cryptococcus neoformans*, fungal diseases, toad.

Since 1993, many amphibian populations have been listed as declining or of conservation concern. Because most amphibians are exposed to terrestrial and aquatic habitats, it is often difficult to determine the factors that affect the ecology and the dynamics of these populations. Many potential disease agents are present in healthy animals and disease can occur when immune systems are compromised (Alford and Richards, 1999).

Cryptococcosis is an important mycosis that occurs worldwide in temperate and tropical climates, and it affects domestic animals and a variety of wild species (Jones et al., 1997). The disease is caused by a soil-inhabiting, yeast-like basidiomycetous fungus, *Cryptococcus neoformans* (Levitz, 1994; Burek, 2001; Travis et al., 2002), which apparently affects immunocompetent and immunocompromised individuals (Buchanan and Murphy, 1998). The disease occurs sporadically and it is not contagious (Burek, 2001), but it has emerged as a major cause of morbidity and mortality in immunosuppressed humans (Buchanan and Murphy, 1998; Travis et al., 2002).

Genetic and biochemical studies have established two varieties of *C. neoformans*: *C. neoformans* var. *gatti* and *C. neoformans* var. *neoformans* (Krockenberger et al., 2003). Most infections in animals are due to the variety *neoformans* (Travis et al., 2002); it is found worldwide, especially in habitats heavily contaminated by bird droppings from rock pigeons (*Columba livia*) or other species (Burek, 2001; Travis et al., 2002). *Cryptoccus neoformans* var. *gatti* has a restricted geographical distribution, mainly subtropical and tropical areas (Jones et al., 1997; Burek, 2001; Krockenberger et al., 2003).

In this article, we report a case of pulmonary cryptococcosis in a free-living adult female toad (*Bufo bufo*) that was killed by a vehicle in northern Portugal. The amphibian was submitted for postmortem examination at the Histopathology Laboratory of the University of Trás-os-Montes e Alto Douro (Vila Real, Portugal). Necropsy examination revealed multiple organ fractures and internal hemorrhages. The lungs had occasional whitish small round soft lesions. No other significant lesions were observed.

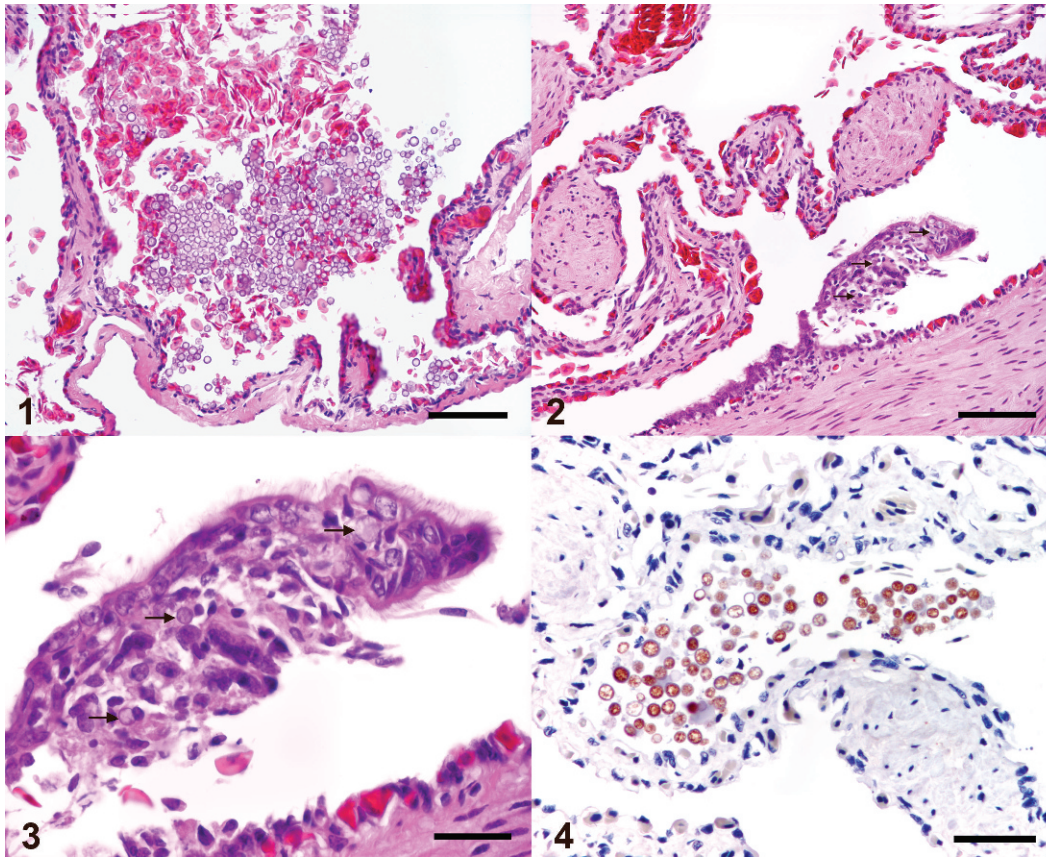

FIGURE 1. Lung. Fungal organisms in the respiratory space, with no inflammatory reaction (PAS). Bar=100 μm. FIGURE 2. Lung. Inflammatory reaction to the fungal organisms (arrows). H&E stain. Bar=100 μm. FIGURE 3. Lung. High-power magnification of Figure 2. Arrows show the fungal organisms. H&E stain. Bar=50 μm. FIGURE 4. Lung. Immunoreactivity of the yeasts to anti-*Cryptococcus* antibody. Streptavidin-biotin-peroxidase, counterstained with hematoxylin. Bar=50 μm.

Organ and tissue samples were fixed in a 10% buffered formalin solution and processed for light microscopy using standard methods. Organ and tissue samples were sectioned at 2 μm and stained with hematoxylin and eosin (H&E), periodic acid-Schiff reaction (PAS), Grocott's methenamine silver stain, and mucicarmin stain. For immunohistochemical characterization, the monoclonal antibody anti-*C. neoformans* was used (clone CSFi, at 1:200; kindly donated by a Neomarkers®, Lab Vision, England, and Inopat, Portugal) and was visualized by a streptavidin and biotin peroxidase technique. This antibody binds to 50 kDa and 90 kDa glycoproteins found on the surface of the yeast wall, and it recognizes encapsulated and acapsular strains of *C. neoformans*.

A polymerase chain reaction (PCR)-based method using a *C. neoformans* species-specific primer was used to confirm the presence of this yeast in tissues. DNA was extracted from the H&E- and PAS-stained sections as described previously (Banaschak et al., 2000; Man et al., 2001), with some modifications. A single multiplex PCR reaction was done using three primers, two universal primers used to identify the presence of any fungal DNA and one *C. neoformans* species-specific primer that allows the detection of this yeast's DNA in clinical samples. This technique has been used at the Medical

Mycology Laboratory at the Institute of Hygiene and Tropical Medicine, Lisbon, Portugal, since 2003 to diagnose cryptococcosis in human patients with infection (Martins, 2001).

Microscopically, both lungs had hemorrhages and various eosinophilic, monomorphic, and spherical to elliptical organisms, measuring 4–27 μm, identified as *Cryptococcus* spp. disseminated in the reticulate infoldings of pulmonic tissue; the yeasts were strongly positive to Grocott's and PAS stains (Fig. 1), but positivity to mucicarmine stain was scarce, because most yeasts were acapsular. The inflammatory response to the agent was minimal, except for an area where the organisms were surrounded by some macrophages and lymphocytes (Figs. 2, 3). The animal also had fatty liver, renal congestion, and ciliated protozoa in the colonic mucosa. Other organs did not have significant alterations.

The identity of the organism was confirmed by immunohistochemistry, using the antibody anti-*C. neoformans* (Fig. 4), and by a PCR-based method (Fig. 5).

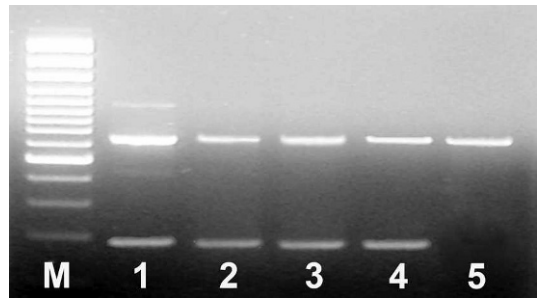

FIGURE 5. Agarose gel electrophoresis of polymerase chain reaction (PCR) products resulting from PCR amplification with three primers: two rDNA Universal delimiting primers led the amplification of a 600-base pair (bp) fragment, and a internal primer specific to *Cryptococcus neoformans* allowed an amplification of a species-specific fragment of 200 bp. M = Marker. 1 = DNA from a pure culture of *C. neoformans*. 2 = DNA from the free-living toad stained tissues. 3 = DNA from blood from a human patient with cryptococcosis. 4 = DNA from cerebrospinal fluid from a human patient with cryptococcosis. 5 = DNA from a pure culture of *Candida* sp.

Pulmonary cryptococcosis is caused by inhalation of spores of *C. neoformans* (Jones et al., 1997; Burek, 2001; Travis et al., 2002). It has a marked propensity for the respiratory system, specially the nasal cavity (Jones et al., 1997; Burek, 2001). The time delay between exposure and clinical disease is highly variable, but there are some reports of acute infection shortly after exposure. Patients with primary pulmonary cryptococcosis rarely have symptoms, or they present nonspecific pulmonary symptoms (Travis et al., 2002).

Diagnosis is based on identification of the organisms by cytology or in tissues, antigen detection assays, and isolation of fungus in culture (Burek, 2001). Although culture is needed for definitive identification, a confident diagnosis can be made by identification of the capsule microscopically (Burek, 2001). Microscopically, the findings are diagnostic, consisting of masses of organisms proliferating with little or no restriction. In H&E sections, the yeast cells are eosinophilic, uninucleate, thin-walled, and spherical to oval (Chandler and Watts, 1988; Jones et al., 1997), measuring 2–20 μm in diameter (mean 4–6 μm). The cells seem to have a faintly stained zone or "halo" that represents a capsule (Levitz, 1994; Burek, 2001; Travis et al., 2002), which stains selectively with mucin stains, such as mucicarmine, PAS, and colloidal iron (Burek, 2001; Travis et al., 2002).

The tissue response to cryptococcal infection depends on the immune status of the patient and whether the organisms have a capsule. When the tissue reaction to the organisms is minimal, as in this case, the air spaces seem filled with myriads of organisms (Travis et al., 2002) and only few macrophages and lymphocytes (López, 2001). A fibrohistiocytic infiltrate may accompany densely packed organisms (Burek, 2001; Travis et al., 2002). Alternatively, organisms may form nodular granulomas similar to other fungal pulmonary infections (Travis et al., 2002).

In conclusion, we describe an appar-

ently healthy animal in which both lungs had organisms, similar in shape and dimensions, to *Cryptococcus* spp., with minimal inflammatory reaction. *C. neoformans* was confirmed by immunohistochemistry and a PCR-based technique. As far as we know, this is the first report of pulmonary cryptococcosis in a free-living common toad.

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