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Cryptosporidiosis Associated with Emaciation and Proliferative Gastritis in a Laboratory-Reared South African Clawed Frog (*Xenopus laevis*)

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A 2-year-old emaciated female South African clawed frog *(Xenopus laevis)* was euthanized because of chronic weight loss. At necropsy, there was no evidence of bacterial, fungal or viral disease; however, the histopathologic findings indicated a proliferative gastritis and the presence of numerous cryptosporidial stages throughout the intestinal tract. Crytosporidial oocysts were present in the water taken from the aquarium housing the infected frog and were likely shed by the sick frog; however, the exact source of the oocysts could not be identified. Water samples from other frog aquaria in the facility did not contain cryptosporidial oocysts. Some *Cryptosporidium* species are important zoonotic pathogens and, to our knowledge, this is the first report of disease associated with *Cryptosporidium* infection in a laboratory *Xenopus laevis*.

Cryptosporidium species are protozoan parasites posing substantial health concerns to animals and human beings (1). Infection with *C. parvum*, for example, one of the most common species in this genus, causes mild to severe watery diarrhea, often accompanied by abdominal pain, fever, vomiting, and weight loss (1, 2). There are increased health risks for infected children, pregnant women, or immunocompromised patients, and the infection can be fatal (2). Water-borne transmission of cryptosporidia is of substantial interest to public health workers. Several recent outbreaks have been reported in the United States, and the suspected causes often include problems with water treatment facilities, poor water filters in swimming pools, or contaminated recreational water (2, 3). In one *C. parvum* outbreak in Wisconsin in 1993, 54 people died and 403,000 became ill (3).

Cryptosporidium parvum has been reported in over 150 species of mammals (4). Other species of *Cryptosporidium* have been reported in fish, birds, and reptiles (5). Some species, such as *C. meleagridis*, lack host specificity (6), and oocysts of many *Cryptosporidium* spp. have great resistance to disinfectants, complicating efforts to control outbreaks (7). The host specificity of cyptosporidial oocytes originating from lower vertebrates (fish, reptiles, and amphibians) and their ability to establish infections among these heterologous groups or in mammals is unresolved. Results of experimental infection studies with mammalian *C. parvum* (8), suggest that infected fish, reptiles, and amphibians could contaminate recreational or drinking water with oocysts that are potentially infective for humans (9). However, experimental infection studies in *Xenopus laevis* indicate that *C. parvum* and *C. serpentis* are not transmissible to this species (10, 11). In addition, to date, there have only been two reported naturally acquired cases of *Cryptosporidium* infection and disease in amphibians: in a Bell's horned frog (*Ceratophrys ornate*) (12) and in an American toad (*Bufo americanus*) (13). We report naturally acquired *Cryptosporidium* infection and disease in a laboratory-reared South African clawed frog, *X. laevis*.

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Case Report

In June 1999, a two-year-old female South African clawed frog (*X. laevis*) was examined because of chronic weight loss and emaciation. The frog, which had been laboratory-reared and purchased from a commercial supplier (NASCO, Madison, Wis.), had been cold-acclimated and housed in the investigator's laboratory for approximately six months in water at 16°C, prior to being transferred to the laboratory animal facility aquaria. Ambient temperature in the animal facility frog aquarium room is kept between 23 and 25°C. Light cycles in the room are kept at alternating 12 h light and 12 h dark. The frog aquaria in the animal facility are 300-L, dark-green, opaque, bathtub-style, self-flushing aquaria, with a water temperature of 19 to 21°C, housing 200 to 300 frogs/aquarium. All frogs in the animal facility, including the affected frog, were fed Purina trout chow three times per week, three hours prior to a filtered water flush from the municipal tap water supply. Fifty percent of the water volume is replaced every three days with tap water passed through a charcoal filter system. The frogs are kept in water with the following water quality parameters: pH 8.7; fluoride, 0.89 ppm; potassium, 0.8 ppm; magnesium, 6 ppm; copper, 70 ppb; chlorine, 0.8 ppm; aluminum < 50 ppb; lead, 2.8 ppb; total coliform bacteria, 0 (% positive samples); and hardness (as $CaCO_3$), 60 ppm. In addition, the Stanford Veterinary Service Center Diagnostic Laboratory monitors the water quality, using a commercial water analysis kit (Voluette Analytical Standards, Hatch Company, Loveland, Colo.). The water quality parameters obtained by use of this water analysis kit (with samples taken at the time this frog was euthanized) were: pH 8.2; water fecal coliform count < 2,000/100 ml; ammonia, 0.25 mg/L; chlorine, 0.10 ppm; nitrate, 0.0 mg/L; nitrite, 0.25 mg/L; and copper, 0.25 g/L. The

aforementioned values were in the "safe" range for aquatic amphibians and were typical for frog water in the animal facility. Animal care and housing were conducted according to recommendations in the NIH Guide for general animal housing and the local, state, and federal fish and game regulations and approved by the institutional committee for animal use and care. The animal facility has maintained an average daily census of approximately 2,000 *X. laevis* under these conditions for the past five years.

According to the investigators, the affected frog had never been experimentally manipulated (e.g., oocytes harvested) and had lost weight over the past six months. On physical examination, the frog was lethargic and was notably thinner than unaffected frogs housed in the same tank, but there were no external findings consistent with trauma, fungal, bacterial, or parasitic disease. The differential diagnosis for chronic weight loss in *X. laevis* includes: parasites; toxicosis due to long-term exposure to high concentration of ammonia, chlorine, nitrates, or heavy metals; neoplasia (which is rare in *Xenopus* sp.); viral disease (which is also rare in *X. laevis*); starvation related to inadequate nutrition; or granulomatous intestinal disease associated with *Mycobacterium* spp. infection (14, 15). Given the poor condition of the frog, it was humanely euthanized by intracelomic injection of 10% tricaine methane sulfonate solution, and the carcass was submitted for necropsy.

Necropsy findings. The adult female frog measured 90.1 cm (snout-to-vent length) and weighed 53.3 g. The frog appeared grossly emaciated. On opening of the coelomic cavity, fat bodies were atrophic and the bright green gall bladder was markedly distended. There were no other abnormal gross findings. Intestinal contents were not sampled for analysis. Tissue specimens from the gastrointestinal tract and all major viscera, as well as sections of skeletal muscle and skin were collected and immersion fixed in neutral-buffered 10% formalin, processed in paraffin, sectioned, and treated with hematoxylin and eosin (H&E) or Giemsa stain for histologic evaluation. In addition, select areas of the stomach were removed from the paraffin-embedded blocks, deparaffinized, and processed for electron microscopy, as described (15).

Examination of the gastrointestinal tract by light microscopy revealed a hyperplastic gastric mucosa with frond-like folds lined by "piled up" epithelium (Fig. 1A). Numerous pale, blue-stained, 0.5- to 3.0-µm, spherical organisms, characteristic of *Cryptosporidium* spp., lined the superficial mucosa, the glands, and were free in the lumen (Fig. 1B). There were no other relevant lesions anywhere in the frog. Histologic evidence of concurrent bacterial or viral (e.g., inclusion bodies) disease was not apparent. Results of microbiological tests on heart blood and liver for opportunistic bacterial pathogens, such as *Aeromonas* spp., *Mycobacterium* spp., and *Flavobacterium* spp., were negative.

Electron microscopy was undertaken to further characterize the parasites (Fig. 2A and 2B). Ultrastructural analysis confirmed preliminary identification of the organism as *Cryptosporidium* sp., and indicated that various life-cycle stages were present. Most prominent were trophozoites attached to the apical border of epithelial cells. Each trophozoite had a large nucleus and nucleolus, and was located above an electron-dense attachment zone, within a parasitophorous envelope (Fig. 2A). Other recognizable stages found in the frog's stomach included first- and second-generation meronts (Fig. 2B), and unsporulated oocysts.

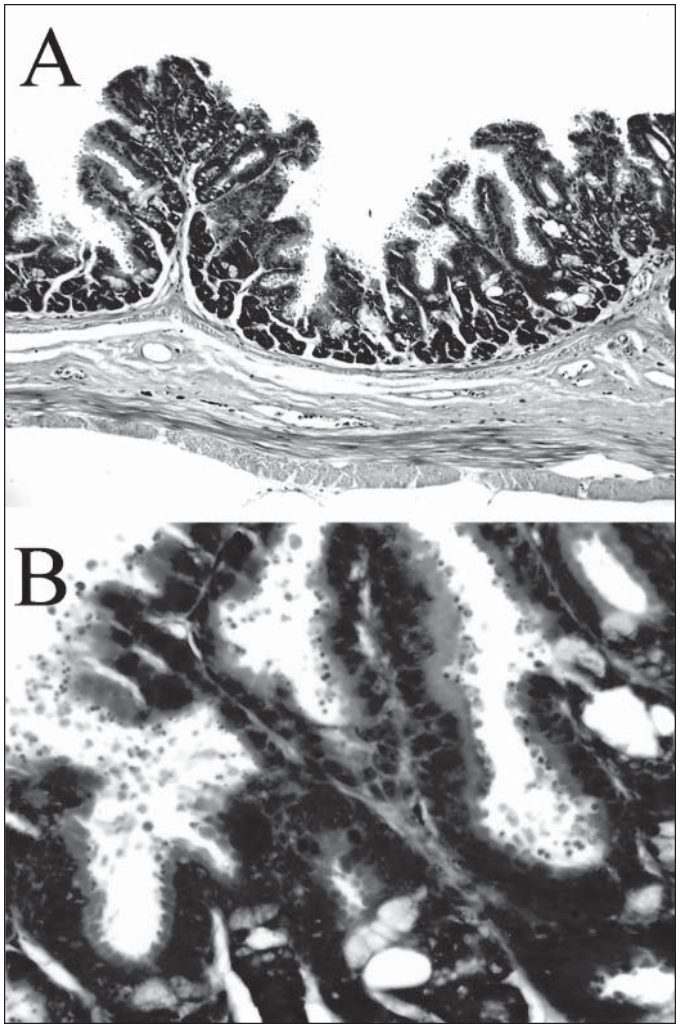

Figure 1. Photomicrographs of the parasitized gastric mucosa from an emaciated laboratory-reared South African clawed frog (*Xenopus laevis*). The gastric mucosa is thick and villus-like (A). Various stages of protozoa line the mucosa are within the epithelial cells (B). Original magnification: A = 10×; B = 40×.

Ancillary diagnostics. On the basis of the histopathologic and electron microscopic findings suggesting infection of the emaciated frog with *Cryptosporidium* sp., formalin-fixed tissues from the affected frog and 40 L of water from the frog's aquarium were collected and sent to the United States Department of Agriculture's Agricultural Research Service in Beltsville, Maryland, where oocysts were cleaned and concentrated from waterborne debris by use of cesium chloride density-gradient centrifugation and were examined by use of bright-field, differential interference contrast, and fluorescent microscopy, according to described methods (16). The concentrated material contained bodies indistinguishable from oocysts of *Cryptosporidium* spp. (Fig. 3A). A small amount of the sedimented material stained positively with MerIFluor fluorescein-labeled anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (Meridian Bioscience, Inc., Cincinnati, Ohio) and was examined by use of fluorescent microscopy. The waterborne oocysts (approx. 2 oocysts/L of water) stained positively with the anti-*Cryptosporidium* reagent, providing further evidence that the oocysts did indeed belong to the genus *Cryptosporidium* (Fig. 3B).

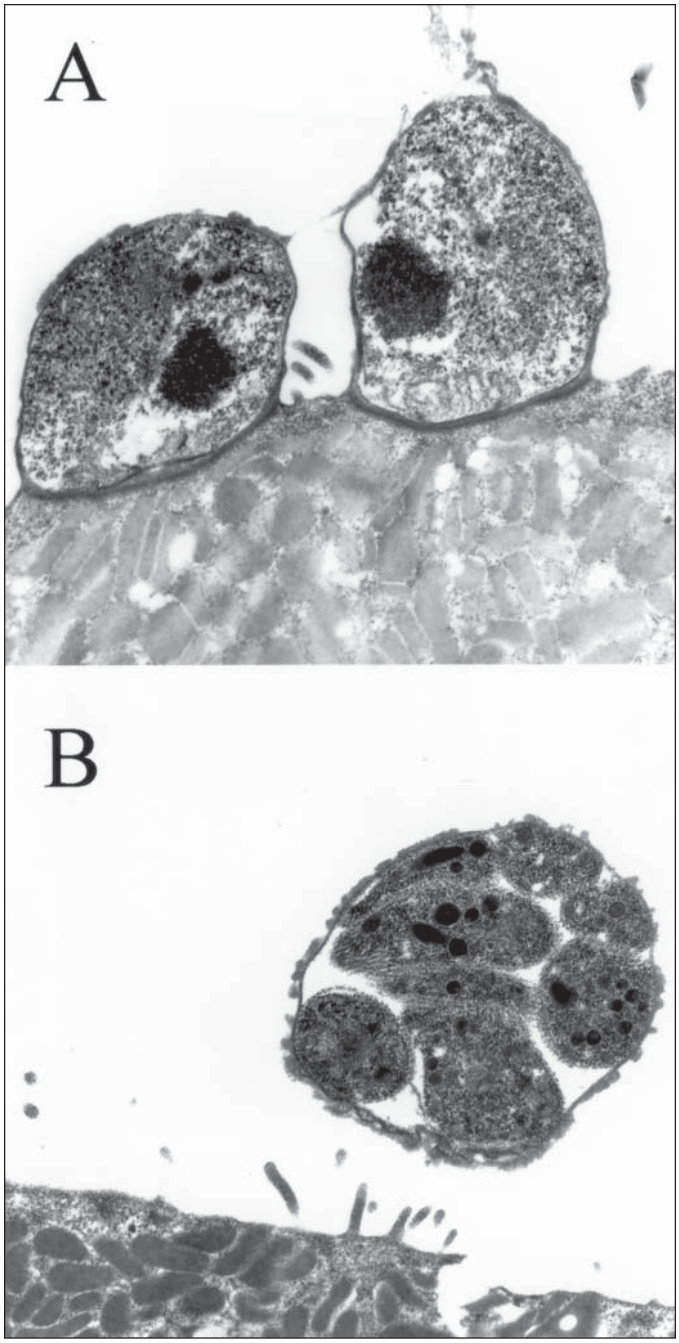

Figure 2. Electron micrographs of *Cryptosporidium* spp. Two trophozoites within parasitophorous envelopes appear to sit on electron-dense attachment zones (A). A schizont containing eight meronts floats above the gastric epithelium (B). Original magnification: A = 13,000×; B = 10,000×.

Discussion

The histologic and ultrastructural morphology of the gastric organisms was consistent with descriptions of *Cryptosporidium* spp. To our knowledge this is the first report of a cryptosporidial infection associated with disease in *Xenopus laevis*. Several studies presented conflicting views on cryptosporidial infections in amphibians. Arcay and coworkers (8, 17) reported naturally acquired and experimentally induced cryptosporidial disease in the cane toad (*Bufo marinus*). However, the organisms are not clearly identifiable as cryptosporidia in the photomicrographs, and the experimental studies lack convincing negative controls. In a study by Graczyk and colleagues (10), poison dart frogs (*Dendrobates auratus*), African clawed frogs (*X. laevis*), dragon lizards (*Pagona vitticeps*), corn snakes (*Elaphe guttata guttata*), and bluegill sunfish (*Lepomis macrochirus*) were directly inoculated (gastrically) with *C. parvum*, but there was no evidence, histologic or otherwise, of gastrointestinal infection in these species. After the inoculation of the *C. parvum*, however, oocysts passed through the gastrointestinal tract and were found in feces of *X. laevis*, but there was no evidence of tissue stages or disease related to the infection (10).

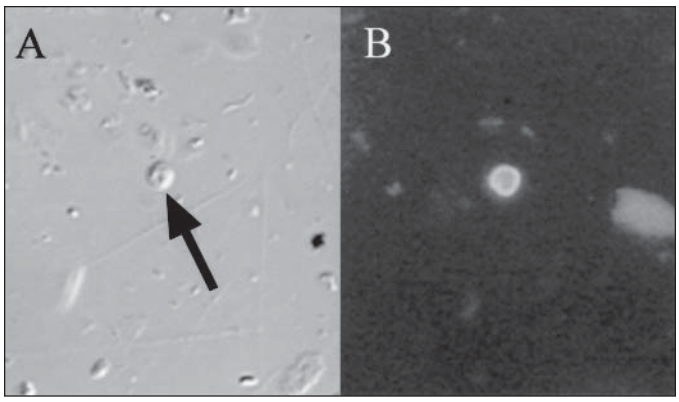

Figure 3. Cryptosporidial-like structures in the water sediment from the affected frog's tank. Bright-field (A) and differential interference contrast microscopy (B). Original magnification of A = 667×. A small amount of the sedimented material from the frog tank water was stained with fluorescein-labeled anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies and examined, using fluorescent microscopy. The water oocysts stained positively with the anti-*Cryptosporidium* reagent. Original magnification of B = 667×.

Results of another study indicated that *C. serpentis* from snakes was not transmissible to *X. laevis* (11). Because tissue forms of *C. parvum* and *C. serpentis* were not found in any of the *Xenopus* frogs in these experimental studies, it has been assumed that amphibians were hosts to other species of *Cryptosporidium*.

The source of infection in the frog in this report is unknown. Since this infected animal was identified, approximately 20 other frogs from the same tank have been necropsied for other purposes, and none were infected with *Cryptosporidium* spp. Testing of water samples collected from all other frog aquaria in the facility did not reveal cryptosporidial oocysts. It is possible that the municipal water supply was contaminated with low numbers of cryptosporidia, but water reports provided by the municipal water testing facility indicated that they were not detected. It is more likely that the affected frog was infected before it was shipped to the animal facility.

Cryptosporidial oocysts are shed in the feces from infected animals and humans into the environment, often a local water source. A potential host then ingests the oocysts, through contaminated food or water, initiating the endogenous cycle in the new host. The life cycle, normally completed in the stomach or intestinal epithelium (depending on the species of *Cryptosporidium*), can also infect the epithelium of the respiratory tract, pancreatic ducts, biliary tracts, pharynx, and gall bladder of immunocompromised hosts (18). Dissolution of a suture in the oocyst wall releases four motile infective forms, the sporozoites that invade epithelial cells. Within the epithelium, they remain

intracellular but extracytoplasmic, appearing by light microscopy to rest on the cell surface and protrude into the lumen. Sporozoites transform into spherical, unicellular trophozoites, which in turn undergo nuclear division and develop by merogony (schizogony) into merozoites. Merozoites leave the parasitized cell and invade other cells, undergoing one or more additional merogonic cycles and eventually forming microgametes (male stage) or macrogametes (female stage). Fertilization of macrogametes is followed by oocyst formation and sporulation within the oocyst to form four sporozoites. The normal function of the epithelium, including defense against the invasion of endotoxins, bacteria, and antigens, is disrupted by the presence of these parasitic stages, either by disrupting the epithelium or by forcing the body to initiate a harmful inflammatory response to the infection, causing atrophy and increased permeability across the epithelium (19). In the gastrointestinal tract, these alterations have the effect of reducing absorption, increasing water secretion, and altering electrolyte homeostasis, which results in clinical diarrhea, malabsorption, and chronic weight loss (19).

It is noteworthy that the *Cryptosporidium*-infected frog of this report was cold adapted at 16°C and was possibly immunocompromised (20). Immunosuppression characterized by lymphopenia and low complement activity are normal physiologic responses of frogs to lower temperatures (20-23). *Cryptosporidium* infections are a serious complication of immunosuppression in immunocompromised humans (2, 16). The additional stress of transport, competition for nutrition, or other factors may have further rendered the frog susceptible to *Cryptosporidium* infection. Cryptosporidiosis should be considered in the differential diagnosis for chronic weight loss in laboratory *X. laevis* and, because it is a zoonotic disease, all laboratory personnel should wear gloves when handling the frogs, water, and equipment.

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