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Case Report

Fatal and disseminated infection by *Entamoeba invadens* in a red-footed tortoise (*Chelonoidis carbonaria*)

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Abstract
Amebiasis is an important parasitosis that can affect reptiles, specially caused by protozoas of the genus *Entamoeba*, which include pathogenic or commensal species. *Entamoeba invadens* is the most common amoeba to cause serious disease and death in reptiles. This paper aims to report a case of a sudden death due to a disseminated infection by *Entamoeba invadens* in a red-footed tortoise (*Chelonoidis carbonaria*). The animal was brought to the Center for Management and Conservation of Wild Animals of the Fauna Division and found dead after being kept in an enclousure with other captive tortoises for 11 months. Macroscopic findings evidenced necrotizing typhlitis and proctitis and round yellow areas in the right lobe of liver parenchyma. In the histological examination, necrotizing and heterophilic enteritis and necrotizing hepatitis with macrovesicular degeneration of hepatocytes, associated with mixed inflammatory infiltrate were present. Both organs revealed numerous amoebic trophozoites, morphologically suggestive of the genus *Entamoeba*, and bacterial colonies. The agent was confirmed by PCR and Sanger DNA sequencing, which leads this study to be the first confirmed case report of *E. invadens* infection in Brazil in a red-footed tortoise.
Keywords: protozoan, amebiasis, trophozoites, reptile, tortoise, hepatitis, enteritis.

Introduction

Parasitic and amphizoic amoebae can cause serious diseases in a huge variety of hosts worldwide. Those frequently reported in humans and animals include the *Entamoeba* complex, *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sapinia* spp. (17, 35, 36). The genus *Entamoeba* comprises endocommensal or endoparasite species, with *Entamoeba histolytica* being the most important one, due to the association with intestinal and extraintestinal infections in humans (15). *Entamoeba dispar*, *E. moshkovskii*, *E. hartmanni*, *E. coli*, *E. polecki*, *Endolimax nana* or *Iodamoeba buetschlii* are also very frequent, but they are usually considered non-pathogenic (33).

In veterinary medicine, two other pathogenic species, highly related to *E. hystolytica*, have been reported. *Entamoeba nuttalli* has been detected in captive and free-living non-human primates, includind macaques, guenon, baboon, colobus, and chimpanzee in zoos (18, 38, 39, 40, 41, 42). *E. invadens* can be responsible for severe disease and death, specially for lizards and snakes, as *Varanus varius* (30), *V. salvator* (31, 6), *V. komodoensis*, *Tiliqua scincoides* (8, 31), *Python molurus bivittatus*, *Boa constrictor* (8), and *Eunectes murinus* (27). Chelonians and crocodilians are usually symptomless carriers and reservoir for this pathogen (2, 9, 22). However, symptomatic cases due to *E. invadens* (12) and *Entamoeba* sp. (26, 28, 20) infections in chelonians have already been reported.

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In Brazil, amebiasis was described in a red-footed tortoise (*Chelonoidis carbonaria*; Spix 1824), but the species of the protozoan had not been determined (29). Therefore, this present report describes, for the first time to the best of our knownledge, a fatal and disseminated infection by *Entamoeba invadens* in a *Chelonoidis carbonaria*.

Case Description

A red-footed tortoise (C*helonoidis carbonaria*) was brought to the Center for Management and Conservation of Wild Animals of the Fauna Division (CeMaCAS) of the Municipality of São Paulo, on June, 2021. At clinical examination, the tortoise was in a good nutritional status, hydrated and had normocolored mucosas. The complete blood count (CBC) revealed mild anisocytosis by polychromasia and microcytosis and mild monocytosis (427 cells/mm³, reference interval 0-376 cells/mm²) (5). No treatment was carried out. The animal was kept in an enclosure with other tortoises in the Rehabilitation Center for 11 months, when it was suddenly found dead. The cadaver was refrigerated and referred to necropsy one day later.

At the post-mortem external examination, the tortoise had 3,330 Kg, with regular body condition and mild nasal and oral liquid brown contents. The animal was hydrated. There was a discrete amount of adipose tissue and serosanguineous liquid in celomatic cavity. The liver was slightly decreased and revealed a round yellow area of 2 cm in diameter, in the right lobe parenchyma. The middle lobe had a firmer consistency and light brown color. The intestines revealed a dark red mucosa, extensive necrotizing typhlitis and proctitis with helminths in its lumen. Other identified alterations were a red lung with moderate amount of brown content at cut and a bladder full of foul odor eritrocromic cloudy urine and red mucosa. The remaining organs had no macroscopic alterations. Coproparasitological exam showed several *Nyctotherus* cysts.

Samples of liver, intestine, kidneys, lung, bladder and heart were collected, fixed in 10% buffered formalin, routinely processed for histopathologic evaluation and visualized with hematoxylin and eosin (HE), Periodic Acid-Schiff (PAS) and Brown Brenn stains. In intestines, the histological analysis exhibited a transmural enteritis with extensively necrosis of the mucosa associated to discrete infiltrate of heterophils, lymphocytes and plasm cells, distributed multifocal to coalescent of all the layers of the organ and an extensive edema of the submucosa and serosa (Fig. 1). In the liver, large and multifocal to coalescent areas of coagulative necrosis associated with thrombotic events were observed, in addition, moderate heterophilic infiltrate in the interface of necrotizing areas and viable parenchyma was present. In the rest of viable parenchyma diffuse severe mixed inflammatory infiltrate, macrovesicular degeneration and multifocal

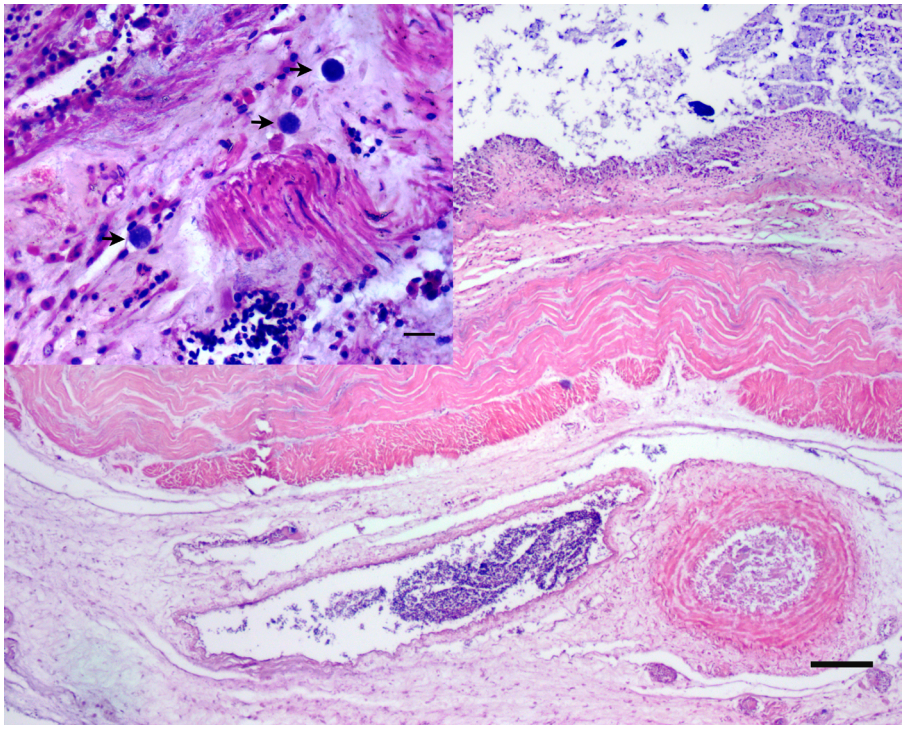

Figure 1. Major: Intestine, HE, 4X. Transmural diffuse enteritis, in the left superior of photography there is l necrosis and detachment of mucosa, in the posterior middle of photograph extensive edema of serosa. Scale bar 100 µm. Inset: Intestine, HE, 40X. Basophilic round structures of *Entamoeba*-like (arrows) in submucosa. Scale bar 10 µm.

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moderate hyperplasia of pigmented macrophages were noted/observed (Fig. 2). There were approximately 10-20 um diameter round structures with granulated to vacuolated basophilic cytoplasm, encompassed by a thick eosinophilic wall and a 5-7 um, round to ovoid and centrally to eccentrally located nucleus containing marginated chromatin, and a slightly basophilic karyosome, positive for PAS stain, in adjacent parenchyma, in both intestine and liver (Figs. 1 and 2 insets). These morphological characteristics were in accordance with the genus *Entamoeba*. Also, gram-negative coccobacilli bacterial myriads were noted in mucosal and submucosal layers in the intestine, inside the parenchyma and blood vessels of liver, heart, kidney and lungs fragments, associated with thrombotic events. Lung and heart fragments showed multiple gram-negative coccobacilli colonies.

DNA from a fragment of fresh frozen liver was extracted with Wizard® Genomic DNA Purification Kit (Promega, Madison, EUA) following the manufacturer's protocol. For amplification, a PCR protocol was performed with primers for the conservative part of SSU rRNA gene specific for the *Entamoeba* genus (Entam1 5'-GTTGATCCTGCCAGTATTATA-TG-3´ and Entam2 5'-CACTATTGGAGCTGGAATTAC-3') (43). PCR conditions were ten minutes at 95°C, 40 cycles of one minute at 95°C, 60°C and 72°C, and final elongation for 10 minutes at 72°C in a Veriti™ 96-Well Fast Thermal Cycler (ThermoFisher Scientific, Waltham, MA, USA). The PCR product were analyzed by agarose gel electrophoresis on a 2% gel and purified with QIAquick PCR Purification Kit (QIAGEN GmbH, Antwerp, Belgium). The Sanger sequencing was carried out using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) and electrophoresed in a the ABI 3500 Genetic Analyzer sequencer (ThermoFisher Scientific, Waltham, MA, USA). BioEdit version 7.2.5 software were applied for analysis and edition. The Basic Local Alignment Search Tool (BLASTn; NCBI https://www. ncbi.nlm.nih.gov/) was used to verify similarity with *Entamoeba* species. The 407 base pair-sequence were deposited in GenBank under accession number OQ255881 and showed 100% of nucleotide similarity with *Entamoeba invadens* (Accession number AY769863.1) isolate from Arakan turtles in 2004, USA. The study was and registered in the National System of Genetic Patrimony Management and Associated Traditional Knowledge (Ministry of Environment, Federal Government, Brazil) - SISGEN (#A3336BC).

Discussion

Entamoeba invadens is the most common pathogenic amoeboid protozoan in reptiles, specially for snakes and lizards (25). Chelonians and crocodilians are usually symptomless carriers and consider reservoirs of this pathogen (14).

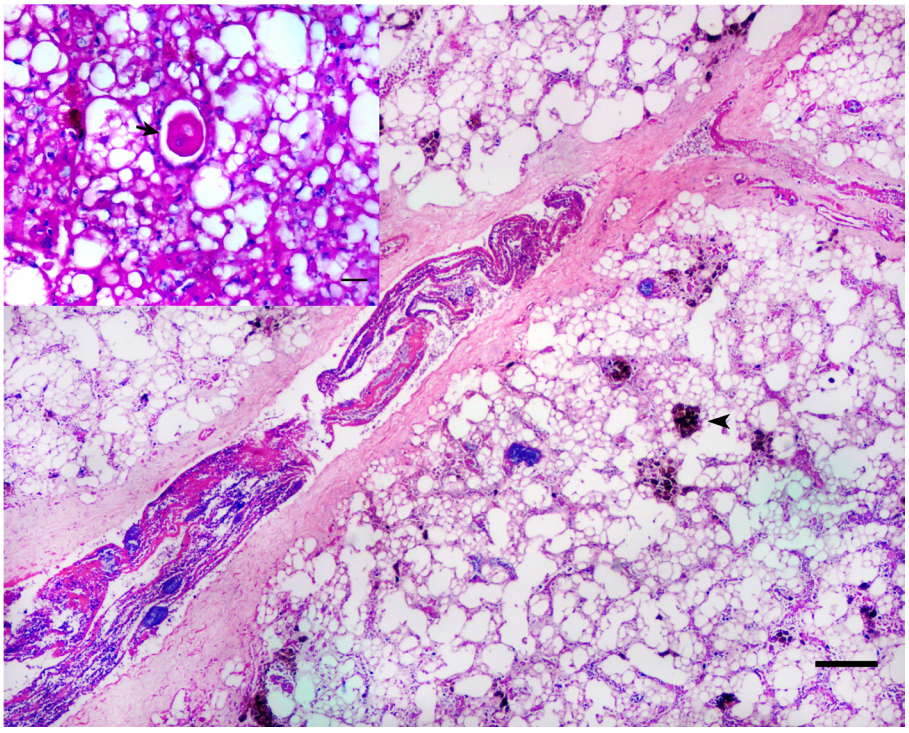

Figure 2. Major: Liver, HE, 4X. Diffuse severe macrovesicular hepatocyte degeneration, bacterial colonies and hyperplasia of pigmented macrophages (arrowhead), associated with mixed inflammation. Scale bar 100 µm. Inset: Liver, PAS, 40X. Round Structure *Entamoeba* like (arrow) in the parenchyma. Note the vacuolated cytoplasm and the centrally to eccentrally located round nucleus. Scale bar 10 µm.

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This is the first report of systemic infection due to *Entamoeba invadens* in a red-footed tortoise (*Chelonoidis carbonaria)* in Brazil.

E. invadens can act as either a gastrointestinal commensal or a pathogen, with a direct life cycle. The transmission occurs by oral-fecal via, when cysts are ingested directly from feces or from a contaminated environment. Excystation occurs within the host intestine releasing the motile trophozoites, which replicate and can either remain within the intestinal lumen feeding on ingesta (20, 44). Amoebisiasis is usually associated to ulcerative lesions in gastrointestinal tract, including stomach, small intestine and colon (6, 16). Invasion can result in embolization of trophozoites to the liver via the portal vein through hematogenous or lymphatic dissemination or ascending the common bile duct to the gallbladder, reaching the adjacent hepatic tissue (14). Widespread dissemination to distant sites as lung, spleen, kidney, and brain (4, 11, 16, 27). Also, a case of necrotizing and granulomatous myositis due to *Entamoeba invadens* had already been described in a common water monitor lizard (*Varanus salvator*) (6). In this case, enteritis and hepatitis were associated with the trophozoites.

The most common presenting complaints in reptiles with amebiasis are decreased appetite progressing to anorexia, regurgitation, dehydration, weight loss and diarrhea, although some individuals present dead or acutely moribund (1, 3, 4, 6, 7, 9, 16, 19, 20, 26, 27). The only clinical sign noticed in this case was weight loss, however other symptoms may not have been identified since this animal lived in an enclosure with other tortoises. *E. invadens* is highly contagious and has caused outbreaks in juvenile (19) and wild-caught chelonians (26), and in captive pythons (16). Pathogenicity is dependent on differences in strain virulence, environmental and host temperature (10, 23, 32, 45) and host conditions, since young, old, immunocompromised, or otherwise debilitated individuals (such as following capture and/or transport) of any reptilian species can develop clinical disease (44). Probably the animal described here could have been immunosuppressed or under stress factors (21). Other tortoises in the same enclosure that died were tested for *E. invadens* by PCR and were negative.

Hematologic findings observed in this case revealed monocytosis, indicating a inflammatory diseases and/or chronic stress in chelonians (28, 37, 46). Hematologic and biochemical abnormalities associated with *E. invadens* are poorly characterized. Reports in a green iguana, in bearded dragon (*Pogona vitticeps*) and in leopard leopard tortoises (*Stigmochelys pardalis*) documented decreased packed cell volume (PCV) with evidence of regeneration, moderate leukocytosis due to heterophilia, monocytosis and lymphopenia, with toxic change and a left shift and mild increase in AST (24, 34).

In affected reptiles, the most commonly necropsy findings included multifocal to coalescing gastrointestinal ulcers, sometimes with concurrent mucosal pseudomembrane formation, edema and hepatomegaly (1, 4, 6, 11, 16, 19, 23, 24, 26, 27). Multiple irregular yellow foci in multiple tissues could be seen in systemic infections (6). Trophozoites can be identified in histologic sections of affected tissues, and are highlighted by PAS (27) and silver stains (1, 16). Other histologic findings include necrosis and hemorrhage with mixed inflammation including heterophils, lymphocytes, macrophages, and eosinophils (1, 16, 26). In the present case, we detected histopathologic lesions that are similar to the reported ones. Direct microscopic examination of saline faecal smear and culture from fresh feces using Robinson's medium could be aaplied for diagnosis, however, false negatives are possible as animals can shed intermittently or have low burden infections (10, 27, 41). PCR and DNA sequencing are sensitive and specific techniques for identifying *E. invadens* in feces (2) and tissues (6, 27). In this case, the molecular examination provide the species of *Entamoeba* involved. It is important to highlight the value of these tests for the definitive diagnosis of *E. invadens*, as only a few reports described the molecular confirmation of this agent, which can be highly pathogenic in reptiles (6, 13, 27, 44). They are relevant tools to corroborate with the epidemiology of *Entamoeba* species.

Treatment for amoebisis is often ineffective and very difficult (10, 20, 26). Therefore, care to avoid potential exposure by limiting mixed-housing of different reptilian species, especially of chelonians with snakes and lizards, is highly recommended, since amebiasis could be a severe infectious disease for tortoises under adverse environmental conditions.

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Conflict of Interest

The authors declare no competing interests.

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