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Fatal *Rameshwarotrema uterocrescens* infection with ulcerative esophagitis and intravascular dissemination in green turtles

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ABSTRACT

The trematode *Rameshwarotrema uterocrescens* (Digenea: Pronocephalidae) parasitizes the glands of the caudal esophagus of *Chelonia mydas*. In the present study, 741 *C. mydas* were examined, 85 animals had adult specimens of *R. uterocrescens* associated with necrotizing ulcerous esophagitis, of these 85, 21 presented invasion of the esophageal mural vessels in the caudal esophagus of juvenile green turtles (*Chelonia mydas*). Necrotizing granulomatous splenitis, hepatitis, and nephritis were associated with the presence of parasites. The eggs from *R. uterocrescens* are birefringent under plane-polarized light, which distinguishes them from those of spirorchiid trematodes. This study contributes novel data on *R. uterocrescens*, methods for detecting this parasite, and demonstrates the fatal potential of parasitism in *C. mydas*.

1. Introduction

Trematodes of the family Pronocephalidae, Looss (1899) have been detected in the alimentary systems of sea turtles, freshwater turtles, and marine iguanas throughout the world (Blair, 2005). Although 12 genera of pronocephalids have been found from sea turtles, few studies have described the pathological changes associated with these parasites. Petechial hemorrhage and ulcerous lesions associated with *Charaxicephaloides* and *Charaxicephalus* have been diagnosed in the gastric mucosa (Santoro et al., 2007a), while *Rameshwarotrema uterocrescens* has been implicated in esophageal lesions (Santoro et al., 2007b; Ribeiro et al., 2017). The present study reports injuries associated with *R. uterocrescens* found in the viscera of juvenile green turtles found in Brazil.

2. Materials and methods

Seven hundred forty one green turtle (*Chelonia mydas*) juveniles from state of São Paulo and the state of Rio de Janeiro (southeastern Brazil) were submitted to necropsy between August 2016 and November 2018. Curved carapace length (CCL) ranged from 30 cm to 37.4 cm and weight ranged from 3 kg to 8.5 kg. Animals used were freshly dead, 85 (11,5%) had *R. uterocrescens* infection, of these 66 (78%) good corporal condition and 19 (22%) were thin.

Systematic necropsy was conducted on all turtles and included histopathology of all organ systems. Tissues were preserved in 10% buffered formalin, processed into paraffin using routine methods, and stained with hematoxylin and eosin and histologic gram stain.

Digeneans were collected from esophagus and liver, spleen and kidney caseous parasitic granuloma, each granuloma having one parasite. Digeneans were placed in 70% ethanol, stained with carmine, and cleared with eugenol. One parasite collected from liver was dissected for eggs measurements. Morphometric data were determined with the aid of a Nikon Eclipse 80i microscope (Kurobane Nikon Co., Ltd., Otawara, Tochigi, Japan) using the NIS Elements BR software program. The digeneans were deposited in the Helminthological Collection of the *Instituto Oswaldo Cruz* (CHIOC 38384) in Rio de Janeiro, Brazil. The key to genera found in Blair (2005) and specific reports by Rao (1975), Santoro et al. (2007a), and Ribeiro et al. (2017) were used for the identification of the parasite and morphometric comparisons.

Prevalence, defined according to Bush et al. (1997), was calculated in the Quantitative Parasitology Program (Qp 3.0; Reiczigel et al., 2005). The 95% confidence intervals (CI) of prevalence were calculated using Stern's exact method.

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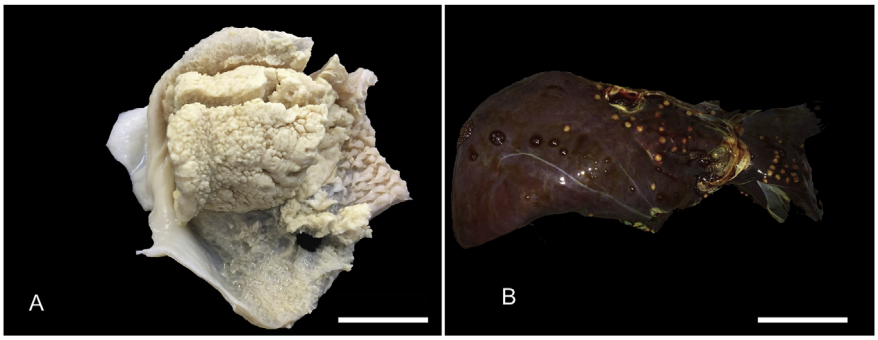

Fig. 1. A Obstructive ulcerous exudative gastroesophagitis, gastroesophageal region, large ulcerated area covered by solid caseous exudate. Scale bar = 3 cm. B Granulomatous necrotic hepatitis, liver, miliary caseous parasitic granulomas. Scale bar = 4 cm.

3. Results

Animals used were freshly dead, 85 (11,5%) had *R. uterocrescens* infection, of these 66 (78%) good corporal condition and 19 (22%) were thin. Post mortem examination in all infected animals, revealed diffuse ulcerations with yellowish necrotic caseous exudate in the caudal esophagus of all infected green turtles. Esophageal obstruction was found in 6 animals (Fig. 1A). Multifocal to coalescing whitish miliary nodules of approximately 3 mm in diameter were found in the liver (15–60 per animal) (Fig. 1B), spleen (5–15), and kidney (1–5) of 21 animals (25%).

Microscopically, the caudal esophagus in all turtles exhibited parasites inside esophageal glands associated with desquamation (Fig. 2A), diffuse necrotizing ulceration covered by an extensive layer of cell debris, degranulated and degenerated heterophils, lymphocytes, plasma cells and parasites (Fig. 2B). Trematodes in the ectatic esophageal glands were surrounded by necrotic mucosa colonized by gram-positive cocci bacterial colonies with marked heterophilic inflammation (Fig. 2C). Parasites were also found in the submucosa and muscle layer of the esophagus as well as lumina of arterioles and venules (Fig. 2 D) of the muscularis and adventitia. Parasitized esophageal blood vessels exhibited the formation of thromboembolisms and mural inflammation consisting of lymphocytes and plasma cells.

In five cases parasites spread to heart (Fig. 2E), lung, and salt gland without inflammation. In spleen, liver and kidney parasites were found associated with parenchyma necrosis with giant cell inflammation manifesting as whitish multifocal randomly distributed caseous granulomas in the kidney (Fig. 2H), liver (Fig. 2F), and spleen. These granulomas were formed by cell debris, degranulated and degenerated heterophils, macrophages, lymphocytes, rare eosinophils, parasites and parasite eggs. The liver and spleen had multifocal groups of five to 10 trematode eggs surrounded by multinucleated giant cells (Fig. 2G). The eggs found in the tissues were oval, had polar processes, and measured between 27.6 μm and 35.6 μm (mean ± SD: 31.2 ± 2.4 μm) in length and between 10 μm and 15.7 μm (mean ± SD: 12.2 + 1.5 μm) in width. Microscopically, the eggs exhibited “shells”, similar to birefringent eggs (Fig. 2H). Inspection under plane-polarized light revealed birefringent trematode eggs within the parasites and all infected tissues. We suspect that others pronocephalids have birefringent eggs but we did not found this information in scientific. However no other parasite with this characteristics and associated with lesions described is know in *C. mydas*.

In all cases specimens of *R. uterocrescens* were measured, 132 specimens were used. The data were expressed as micrometers (mean ± SD). The parasites were small, measuring 1.074 ± 111 mm (999–1202 μm) long and 470 ± 57 μm (407–519 μm) wide, tapered in the anterior region and rounded in the posterior regions, without cephalic collar. The terminal oral suckers measured 82 ± 9 μm (74–92 μm) in length and 111 ± 2 μm (61–77 μm) in width. The pretesticular ovary measured 69 ± 6 μm (63–76 μm) in length and 66.9 ± 9 (61–77 μm) in width. Two large testes occupied the posterior extracecal region of the body posterior to the vitelline follicles. The right teste measured 163 ± 28 μm (145–196 μm) in length and 118 ± 4 μm (84–164 μm) in width. The left teste measured 175 ± 14 μm (159–188 μm) in length and 107 ± 44 μm (62–150 μm) in width. Vitelline follicles were located in the lateral portion of the body, anterior to the ovary. Uterine loops extended to the lateral edge of the body and the cecum terminated posterior to the testes (Fig. 3A). The eggs recovered from the dissected specimen had a length between 28 and 32 (mean ± SD: 29 ± 1.5 μm) and width between 16 and 19 (mean ± SD: 17 ± 0,8 μm) with evident polar processes (Fig. 3C).

4. Discussion

Among the 741 turtles examined, 85 (11.52%, CI = 9.3–14.0) were positive for esophagus lesions. Among the positive hosts, 21 (24.7%, CI = 16.3–35.2) had systemic parasitosis.

Reports of lesions due to infection by *R. uterocrescens* have described the occurrence of the parasite in esophageal glands and muscle layers (Santoro et al., 2007a, Ribeiro et al., 2017). Its presence in arteries and veins means that the circulatory system is the pathway to the spleen, liver, kidneys, and other organs (Wyneken, 2001). We suspect that the parasite first weakens the lining epithelium of the esophagus and esophageal glands, leading to the establishment of opportunistic esophageal bacteria. The large amount of necrotic debris in the lesions found in the esophagus of the green turtles was associated with secondary bacterial colonies as well as the heterophilic inflammatory response. In the esophagus, the necrosis caused by bacteria reached the submucosal and muscle layers, eventually exposing blood vessels to the parasites. The significant association between the lesions and bacterial colonies in the esophagus, makes infection by *R. uterocrescens* always potentially fatal.

The granulomatous hepatitis and splenitis caused by *R. uterocrescens* eggs are compatible with findings discussed in a previous study describing the giant cell inflammatory response involving *R. uterocrescens* eggs in esophageal wall from a juvenile *C. mydas* found on the coast of the state of Santa Catarina (southern Brazil) (Ribeiro et al., 2017).

Parasites inside blood vessels in the present study were initially considered to be associated with infection by digeneans from the family Spirorchiidae due to the similarities in the findings as reported for sea turtles on the Brazilian coast (Werneck et al., 2016). Nevertheless, the occurrence of pale, round, birefringent eggs in the tissues and inside parasites (observed under plane-polarized light and subsequently see in eggs found in the uterus of the parasites) confirmed the notion that *R. uterocrescens* was involved in all the processes reported in the present study. Therefore, plane-polarized light is a useful resource for differentiating spirorchiid eggs from *R. uterocrescens* eggs in granulomatous processes in the tissues of sea turtles.

Rameshwarotrema uterocrescens was first described using specimens found in the intestines of *C. mydas* in the Manar Gulf, India (Rao, 1975).

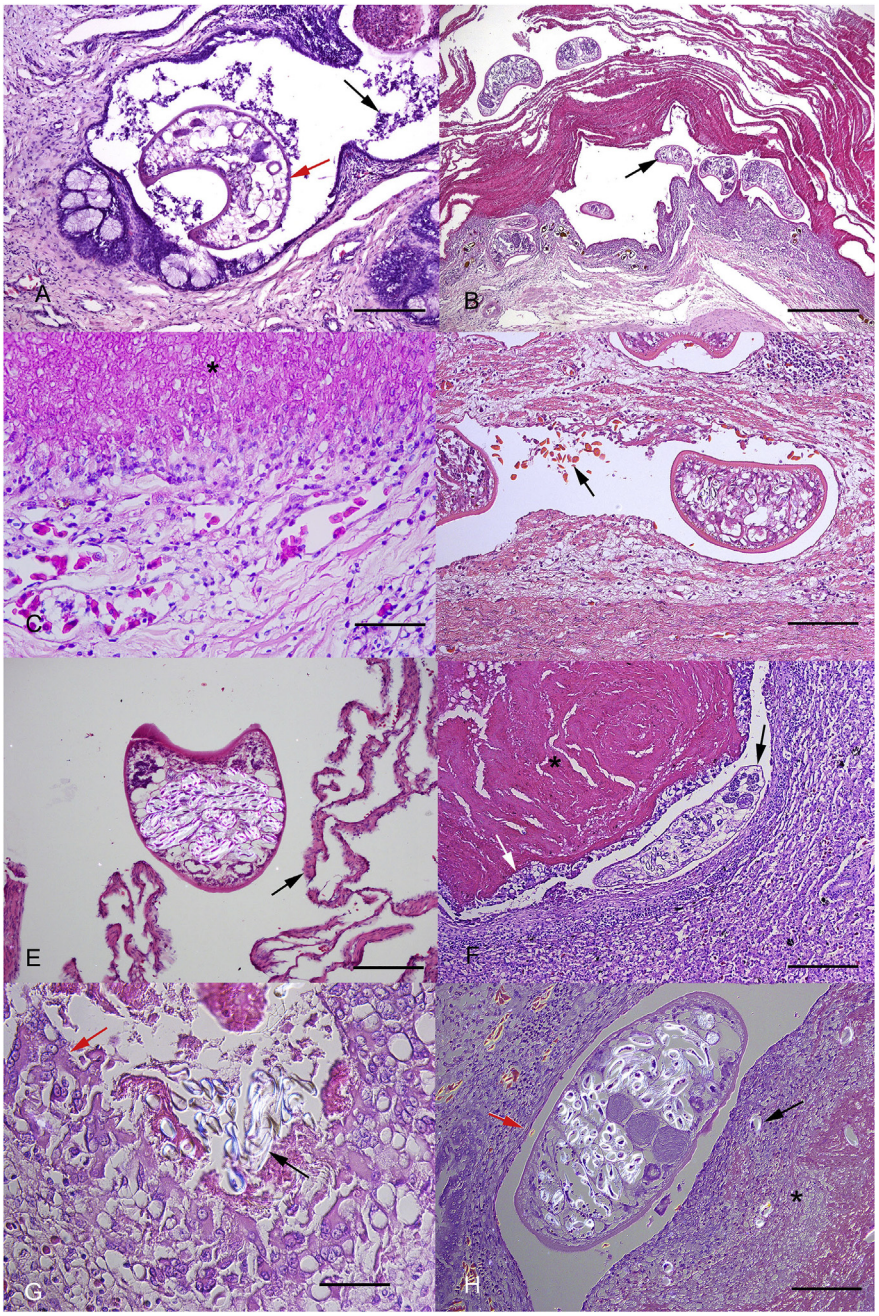

Fig. 2. A Initial lesion caused by *R. uterocrescens* (red arrow) associated with esophageal gland desquamation (black arrow). Scale bar = 100 µm.
B Ulcerative esophagitis, esophagus, extensive loss of esophageal mucosa with eight specimens of *R. uterocrescens* (arrow) embedded in necrotic amorphous eosinophilic tissue in submucosa with marked heterophilic inflammatory infiltrate. Scale bar = 500 µm.
C Marked inflammation with heterophils and macrophages (*) in esophageal submucosa. Scale bar = 50 µm.
D. *R. uterocrescens* in ectatic vessel, note red blood cells (arrow). Scale bar = 100 µm.
E Heart with *R. uterocrescens* under plane-polarized light, with birefringent eggs between myocardiocytes (arrow). Scale bar = 100 µm.
F Granulomatous hepatitis, liver, *R. uterocrescens* (arrow) next to necrotic mass (*) formed by degenerate leukocytes, rare parasite eggs, cell debris peripherally enveloped by multinucleated giant cells. Scale bar = 200 µm.
G Granulomatous hepatitis, liver, birefringent *R. uterocrescens* eggs (black arrow) under plane-polarized light enveloped by multinucleated giant cells (red arrow). Scale bar = 50 µm.
H Immersed eggs (black arrow) in thrombotic (*) arteritis (red arrow). Parasite in kidney artery seen under plane-polarized light with intensely birefringent eggs. Scale bar = 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Further studies discussed the occurrence of specimens of *R. uterocrescens* in the intestinal mucosa of two *Eretmochelys imbricata* individuals from Puerto Rico (Dyer et al., 1995) and the esophagus of 10% of 47 adult green turtle females in Costa Rica, with mean abundance of 0.6 ± 1.8 and mean intensity of 5.7 ± 1.9 (range: 3 to 7) (Santoro et al., 2006). A study of 136 *C. mydas* juveniles found along the Brazilian coast detected *R. uterocrescens* in 25 animals, with prevalence of 18.4 (confidence interval [CI]: 12.5 to 26.0), mean abundance of 4.35 (CI: 1.67 to 13.5), and mean intensity of 23.7 (CI: 9.84 to 70.4) (Werneck and Silva, 2015).

In the present study, the morphological analysis revealed that the animals did not exhibit a cephalic collar. Other traits, such as the shape and location of vitelline follicles and teste, uterine loops, and ceca, are compatible with the identification key of the genus and the original description of the species (Blair, 2005; Rao, 1975; Ribeiro et al., 2017).

The eggs collected from the ruptured specimen had polar processes (Fig. 3C), these processes were not described by Rao (1975) or Ribeiro et al. (2017). These processes were not visualized in the eggs in the uterus of intact flukes (Fig. 3 A-B).

5. Conclusion

Infection by *R. uterocrescens* in juvenile green turtle is fatal affecting a substantial number of individuals. It caused serious ulcerous necrotizing esophagitis with secondary bacterial infection and sometimes total esophageal obstruction. This is the first study to describe extra-esophageal lesions associated with *R. uterocrescens*, confirming the spread of the parasite causes severe granulomatous hepatitis, splenitis and nephritis. Hematogenous spread of parasites was associated with miliary granulomatous hepatitis, splenitis and nephritis because parasites were found in these lesions. Moreover, plane-polarized light is a useful resource in the diagnosis of this parasitosis.

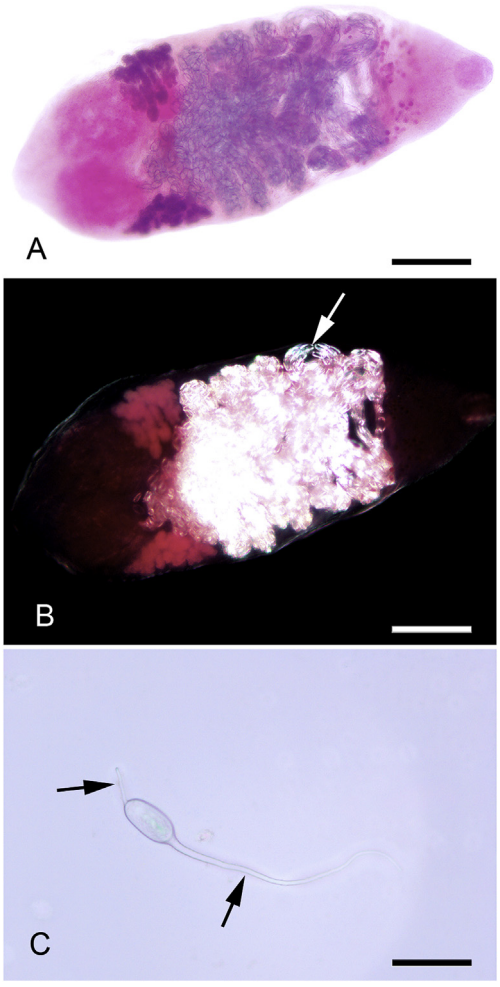

Fig. 3. A *Rameshwarotrema uterocrescens* Rao (1975) (Digenea, Pronocephalidae) from *Chelonia mydas* Linnaeus 1758 (Testudines, Cheloniidae) from Brazil. Scale bar = 200 μm.
B *Rameshwarotrema uterocrescens* Rao (1975) (Digenea, Pronocephalidae) from *Chelonia mydas* Linnaeus 1758 (Testudines, Cheloniidae) from Brazil under plane-polarized light. Note birefringent eggs (arrow). Scale bar = 200 μm.
C Egg dissected from *Rameshwarotrema uterocrescens* Rao (1975) (Digenea, Pronocephalidae) from *Chelonia mydas* Linnaeus 1758 (Testudines, Cheloniidae) from Brazil. Note polar filament (arrow). Scale bar = 50 μm.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.06.006.

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