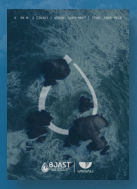
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SYSTEMIC MYCOBACTERIOSIS IN DANIO RERIO (ZEBRAFISH): CLINICAL AND HISTOPATHOLOGICAL FINDINGS

Silva, D. L. O.*[a], Giesta, L. B.[a], Hoefling, L. C.[a], Romano, L. A.[a], Pedrosa, V. F.[a]

[a]Laboratório de Imunologia e Patologia de Organismos Aquáticos, Instituto de Oceanografia, Universidade Federal do Rio Grande – FURG, Rio Grande, Brasil.
*dileandro10silva@gmail.com (Corresponding author)

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ABSTRACT

This case report describes systemic mycobacteriosis in Danio rerio from the FURG zebrafish facility, highlighting the clinical manifestations and histopathological findings that confirmed infection by *Mycobacterium* spp. Gross examination revealed nodular and ulcerative skin lesions, while internal evaluation indicated widespread inflammatory involvement consistent with chronic infection. Histopathology showed well-organized granulomas in multiple organs, including the spleen, liver, kidney, and skeletal muscle. These lesions exhibited a caseous necrotic core surrounded by lymphocytic infiltration, abundant epithelioid macrophages, and multinucleated giant cells, characterizing the typical granulomatous response associated with mycobacteriosis. To identify the etiological agent, different staining techniques were applied; the Fite-Faraco method was selected due to its higher sensitivity for detecting non-tuberculous *Mycobacterium* species, which contain lower levels of mycolic acids in their cell walls. This technique revealed numerous acid-fast bacilli within the granulomas across all examined tissues, confirming the diagnosis of systemic mycobacteriosis.
The findings reinforce the chronic and multisystemic nature of the disease, and its importance as both a sanitary and zoonotic concern. Mycobacteriosis is associated with significant economic losses, mortality, and occupational risks, especially in research facilities and aquaculture systems lacking adequate biosafety measures. Furthermore, the study highlights the central role of histopathological diagnosis combined with special staining techniques for accurate detection of the pathogen. Overall, these cases emphasize the need for continuous surveillance and improved management practices to prevent the dissemination of *Mycobacterium* spp. in laboratory and aquaculture environments.

Keywords: *Mycobacterium* spp. Zebrafish, Granulomas. non-tuberculosis mycobacteria. Fite-Faraco.

1 Introduction

Danio rerio is a teleost fish species widely used in biomedical research, toxicological studies, and aquarium keeping. Its frequent use is due to its small size, ease of maintenance, and genetic similarity to humans (Suurväli et al., 2020; Aksoy et al., 2019). These characteristics allow researchers to study biological processes and diseases efficiently and on a large scale, including infectious diseases such as mycobacteriosis.

Mycobacteriosis in fish is a chronic disease caused by bacteria of the genus *Mycobacterium*, family Mycobacteriaceae (Tortoli, 2019). The main species associated with infections in fish are *Mycobacterium marinum* and *M. fortuitum*.

Mycobacteria are slightly curved, rod-shaped, non-motile bacilli characterized by their resistance to acid-alcohol staining after Ziehl-Neelsen staining. They are Gram-positive, non-spore-forming organisms capable of withstanding a wide range of environmental temperatures and are notably resistant to antibiotics and disinfectants (Ziehl, 1882; Falkinham, 2021).

The disease typically causes multisystemic damage, leaving affected fish lethargic, displaying erratic swimming, and unable to feed (Giavenni et al., 1980). As part of the host's defense response, granulomata develop (Foey, A. & Picchietti, S., 2014). A granuloma is an organized inflammatory structure formed to isolate and contain the pathogen's development (Noga et al., 1989).

A key indicator of chronic inflammation in mycobacteriosis is the presence of multinucleated giant cells, which arise in an attempt to contain intracellular agents that are difficult to eliminate, such as mycobacteria (Davis & Ramakrishnan, 2009). Therefore, granulomas are considered the typical histopathological feature of the disease (Polinas et al., 2021).

This report describes cases of systemic mycobacteriosis in *Danio rerio*, highlighting the clinical manifestations and histopathological findings. Although it is known that mycobacteriosis affects several species of fish and is not uncommon, the methodology presented in this study, is a simple, accessible technique capable of providing an accurate diagnosis.

Given that the disease affects aquaculture production and poses risks to human health, mycobacteriosis is considered a zoonosis. Transmission is associated with inadequate husbandry practices and poor sanitary conditions, typically occurring through direct contact with infected fish or contaminated water (Haenen et al., 2013; Hashish et al., 2018). In addition to the zoonotic risk, bacterial infections cause significant economic losses and fish mortality (Lafferty et al., 2015).

Six zebrafish from the FURG bioterium were submitted to the FURG Laboratory of Pathology and Immunology of Aquatic Organisms (LIPOA) with skin lesions suggestive of mycobacteriosis. Histopathological analyses were requested from the Department of Physiological Sciences of the same institution, to confirm the diagnosis.

Fish necropsies were performed according to the protocol established by (Brenner & Home, 1959). After euthanasia, skin and muscle tissues were dissected and placed in histological cassettes. The samples were then fixed in 10% buffered formalin. All tissues were processed on a LEICA TP 1020 processor and embedded in Paraplast. Blocks were sectioned at 5 µm using a LEICA RM 2245 microtome.

Histological sections were stained with hematoxylin and eosin (H&E), PAS, Gram, Giemsa, and Fite-Faraco. Photomicrographs were obtained using a Zeiss microscope equipped with an AxioVision camera and ZenLite software.

Macroscopic examination revealed nodular lesions with an ulcerative appearance (Figures 1 and 2). A marked inflammatory reaction was observed throughout the peritoneum, with abundant lymphocytes, macrophages, and epithelioid cells surrounding a necrotic, caseous center. The lesions extended to the spleen, liver, kidney, and skeletal muscle (Figure 3).

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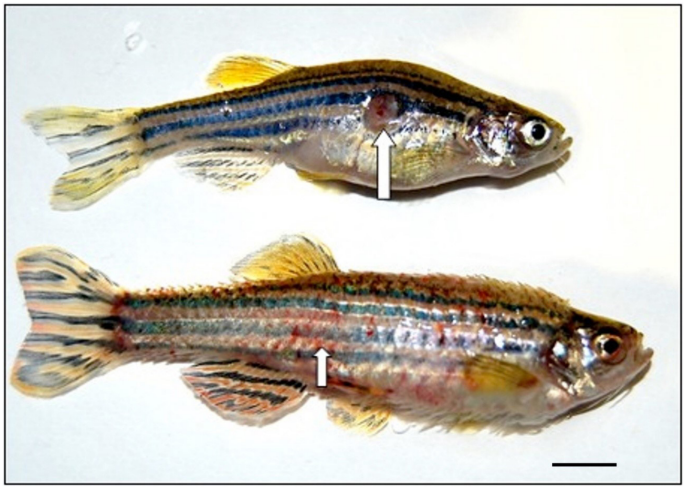


Figure 1. Two specimens of *Danio rerio* with ulcerative skin lesion (long arrow), H&E (short arrow), and erect scales. BAR: 1.5cm

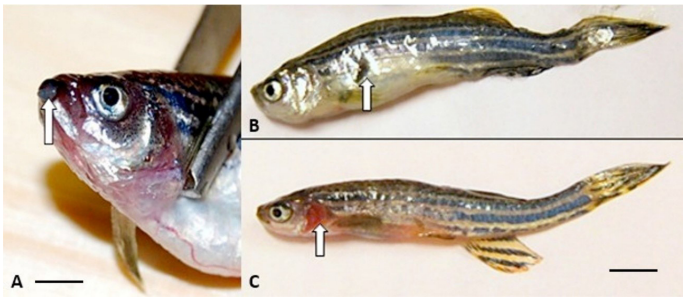


Figure 2. Macroscopic lesions in a zebrafish (*Danio rerio*) specimen: A - Millimetric brown nodular lesion in the buccal region (arrow). B - Ulcerative lesion on the flank (arrow). C - Opercular agenesis (arrow). Bar: A – 1cm; B e C – 1.7cm.

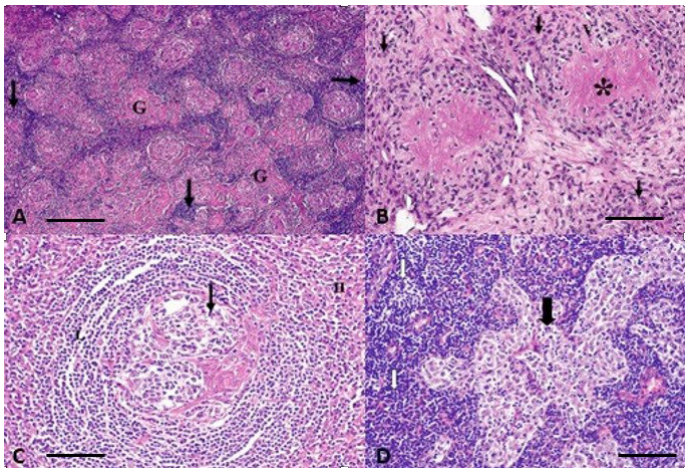


Figure 3. Photomicrographs of zebrafish (*Danio rerio*) liver tissue: A - Multiple granulomas (G) surrounded by abundant inflammatory tissue (arrows). H&E 10x; B - Granulomas with central caseous necrosis (*), inflammatory infiltrates, and fibroblastic proliferation (arrows). H&E 40x; C - Granuloma with lymphocytic corona (L) and central epithelioid cells (arrow). Hepatic remnants are visible peripherally (H). H&E 40x; D - Inflammatory tissue with abundant epithelioid cells (black arrow) and a marked lymphocytic reaction (white arrows) 40x. BAR: A – 100µm; B – 50µm; C – 50µm; D – 100µm.

Differential staining techniques were used to characterize the etiological agent. H&E staining revealed a granulomatous inflammatory pattern, but specialized stains were required to identify the pathogen. The Fite-Faraco (FF) technique, specifically adapted for *Mycobacterium* spp in fish due to its lower mycolic acid content, was selected to preserve the lipid-rich cell wall and enhance detection. FF staining confirmed the presence of abundant acid-fast bacilli (AFB) in the granulomas of all affected organs, establishing the diagnosis of systemic mycobacteriosis. Acid-fast bacilli consistent with mycobacteria were found in all lesions. Mycobacteriosis was therefore diagnosed in the liver and skeletal muscle based on the histopathological evaluation (Figures 4 and 5).

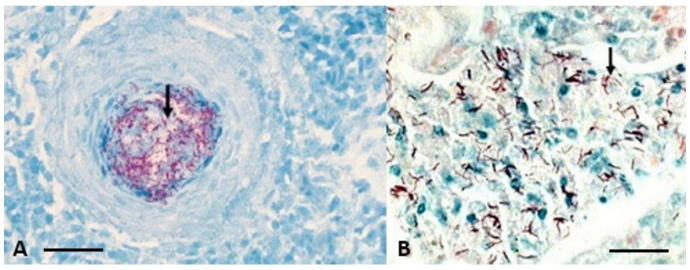


Figure 4. Photomicrograph of zebrafish (*Danio rerio*) liver tissue: A- Granuloma containing multiple acid-fast bacilli stained pink (arrow) against a blue background. Fite-Faraco 40x; B- Detail of acid-fast bacilli (arrow). Fite-Faraco 100x. BAR: A – 20µm; B - 10µm.

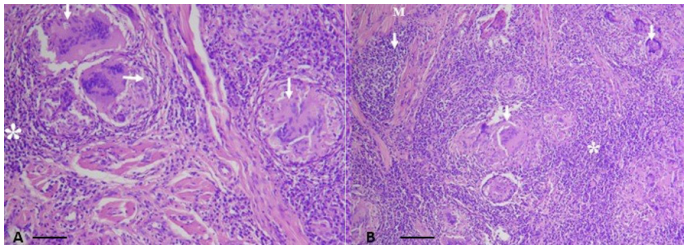


Figure 5. Photomicrographs of skeletal muscle tissue from zebrafish (*Danio rerio*): A- Granuloma with multiple multinucleated giant cells (arrows) and peripheral lymphocytic reactivity (*). H&E 20x; B- Multiple granulomas with abundant multinucleated giant cells (arrows), reactive lymphoid infiltrate (*), and skeletal muscle infiltration (M). H&E 10x. BAR: A - 50µm; B - 100µm.

Mycobacterium spp is an acid-fast bacterium with a lipid-rich cell wall that produces mycolic acids. Non-tuberculous mycobacteria have lower mycolic acid levels compared to tuberculous species. To identify the bacteria, we used the special Fite-Faraco technique, which is preferred for mycobacteria infecting fish because these species contain reduced concentrations of mycolic acid in their cell walls. The FF technique better preserves the lipid layer, which is essential for accurate visualization of acid-fast organisms (Romano et al., 2020).

The examinations performed in this study revealed acid-fast bacilli, confirming the presence of non-tuberculous mycobacteria. Granulomas containing multinucleated giant cells and central caseous necrosis were also observed.

Sanlund, et al. (2023), in a study of Atlantic mackerel (*Scomber scombrus*), described well-defined spherical granulomas with a necrotic core, primarily in the kidney. Swaminathan, et al. (2021), studying goldfish (*Carassius auratus*), observed granulomas in the kidney and spleen, along with cutaneous fibroma. In the present case, we identified a marked inflammatory reaction and granulomatous lesions in the spleen, liver, kidney, and muscle, in addition to systemic involvement of multiple visceral organs.

These findings corroborate the systemic nature of mycobacteriosis, a pattern frequently reported in *Mycobacterium* spp. infections in fish (Ishikawa, et al., 2001; Gauthier, & Rhodes, 2009).

The granulomas observed in this study, composed of epithelioid macrophages and multinucleated giant cells, also exhibited a crown-like lymphocyte infiltrate. This indicates a healing process associated with chronicity and reflects the organism's attempt to contain the pathogen (Lequain, et al., 2023).

The distribution of the lesions and the systemic characteristics documented here are consistent with findings in other species, demonstrating the broad pathogenic potential of the agent. In Mullet (*Mugilidae*), for example, *M. marinum* was identified as the cause of mycobacteriosis, with the spleen being the most affected organ (Antuofermo, et al., 2017). In zebrafish (*Danio rerio*), experimental infections with *M. marinum* strains produced peritonitis and granulomas in multiple organs, while *M. chelonae* and *M. abscessus* caused granulomas in visceral organs in humans (higher mammals) (Watral & Kent, 2007).

The reported cases of systemic mycobacteriosis in *Danio rerio* highlight the importance of histopathological diagnosis in identifying chronic bacterial infections in fish. The granulomatous lesions observed, together with the acid-fast bacilli detected by Fite-Faraco staining, confirm infection by *Mycobacterium spp*.

Understanding the inflammatory response and pathogenic mechanisms involved is essential to prevent outbreaks and minimize production losses, contributing to improved knowledge of chronic bacterial diseases in aquatic organisms. Mycobacteriosis represents both a health and zoonotic challenge and requires strict biosafety measures for effective control in research and aquiculture environments.

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