NOTE

Mycobacterium fortuitum infection in silver arowana (*Osteoglossum bicirrhosum*)

K. Varello[1*], M. Prearo[1], E. A. V. Burioli[2], P. Pastorino[3], D. Mugetti[1], S. Meistro[1], M. Righetti[1], E. Bozzetta[1]

[1] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148 – 10154 Turin, Italy; [2] LABÉO Frank Duncombe, 1 route de Rosel – Saint Contest – 14053 Caen, France; [3] Dipartimento di Scienza della Vita, Universita degli Studi di Trieste, Via L. Giorgieri 10 - 34127 Trieste, Italy

Abstract
A case of mycobacterial infection in silver arowana (*Osteoglossum bicirrhosum*) is described in the present work. An adult male silver arowana exhibited granulomas in the liver and spleen with acid-fast bacilli identified as *Mycobacteria fortuitum*. To our knowledge this is the first report of mycobacterial infection in this fish species.

The silver arowana, *Osteoglossum bicirrhosum* (Cuvier, 1829), is a tropical freshwater fish of the family Osteoglossidae also named "bonytongues" (Allen et al., 2002). This species is native of the rivers in the South American tropical area, near the Rio de Janeiro and Orinoco basins (Maldonado-Ocampo et al., 2008).

In the Amazon region this species has an economic significance as a food fish (Garcia et al., 2009) but in other countries it is widely marketed in the aquarist sector (Tovar Verba et al., 2014).

Non-tuberculous mycobacteria (NTM) are worldwide recognised as causative agents of infections in fish, also transmissible to human beings. The species most frequently isolated are *Mycobacterium marinum, Mycobacterium fortuitum* and *Mycobacterium chelonae* (Decostere et al., 2004; Gauthier and Rhodes, 2009), however many other species have been isolated in fish both with and without symptoms such as *M. abscessus, M. chesapeaki, M. shottsii, M. pseudoshottsii, M. gordonae, M. peregrinum, M. salmoniphilum* and *M. nonchromogenicum* (Heckert et al., 2001; Rhodes et al., 2004, 2005; Righetti et al., 2014; Puk et al., 2018).

M. marinum, *M. chelonae* and *M. fortuitum* are also the species most frequently associated with human infections (Decostere et al., 2004) to which *M. abscessus* has recently been added to the previous ones (Lee et al., 2010).
In ornamental fish, the presence of NTM has been reported and monitored for decades, and

* Corresponding author's email: katia.varello@izsto.it

it continues to be an issue of great interest for its importance with regards to zoonosis (Zanoni et al., 2008; Puk et al., 2018).

No cases of mycobacterial infections in fish of the family Osteoglossidae have been reported to date. The aim of the present work is to describe the first case of mycobacterial infection in a silver arowana.

An adult male silver arowana, reared individually in a large size private aquarium (800 L), was found dead and sent for post-mortem investigations at the Fish Diseases Laboratory of the IZS PLV (Turin, Italy).

The animal was necropsied under aseptic conditions and examined for lesions. Samples from liver, spleen and kidney were collected for histopathological, parasitological and bacteriological investigations, including mycobacterial culture, according to the standard protocol applied for ornamental fish in the laboratory.

The tissues for histopathology were fixed in 10% neutral-buffered formalin and processed by standard paraffin wax techniques. Samples were cut in 4±2 μm sections and stained with haematoxylin-eosin (HE) and Ziehl-Neelsen (ZN) methods for acid-fast bacilli. The slides were evaluated microscopically at increasing magnifications (100x, 200x, 400x); diagnosis of mycobacteriosis was based on the observation of granulomatous lesions in selected organs associated with acid-fast rods.

Standard bacteriological examination was performed from kidney tissue using first isolation media (Columbia Blood Agar - CBA). The colonies grown after 24-48 h of incubation at 22±2°C were selected, cloned to pure culture on CBA and identified by biochemical tests (API 20E and 20 NE, bioMérieux, France).

For mycobacterial culture, fresh tissues collected from spleen and liver were homogenised separately and decontaminated for 30 min using 1.5% cetylpyridinium (Sigma-Aldrich, Italy). Ten μL of each homogenate were spread on a glass slide, over an area of approximately 1 cm x 2 cm, and then, stained using the ZN method. At least 300 fields were microscopically examined at high magnification (1000x). One loop of 10 μL of the decontaminated homogenate was inoculated on 2 Stonebrink medium (Microbiol, Uta, Cagliari, Italy) tubes and 2 Löwenstein-Jensen medium (Microbiol, Uta, Cagliari, Italy) tubes. For each medium, one tube was incubated at 30±1 °C and the second at 25±1 °C. The tubes were checked daily for 2 months; all suspected colonies were microscopically examined using ZN staining and were also subcultured. All isolates were identified following the methods of Kent and Kubica (1985) and Wayne and Kubica (1986).

Bacterial strains isolated from both the spleen and liver were suspended in 200 μL of DNAase-free water and subjected to DNA extraction using a QIAamp DNA minikit® (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol for blood or body fluids, except for elution performed in 60 μL Qiagen elution buffer AE. Extracted DNA was subjected to amplification and sequencing of the *hsp65* gene, using the primer pair Tb11-Forward (5'ACCAACGATGGTGTGTCCAT3') and Tb12-Reverse (5'CTTGTCGAACCGCATACCCT3') (Telenti et al., 1993). Forward and reverse sequences were aligned using ClustalW. *hsp65* sequences were analysed using BLAST® tool, based on NCBI database.

At necropsy, no visible lesions neither cutaneous nor in the visceral organs were observed. At histopathological examination, granulomatous lesions were detected in the liver and the spleen (Figure 1) but not in the kidney. Granulomas showed a central necrotic area surrounded by several layers of laminar necrotic material and externally enclosed by flattened spindle-shaped cells. All granulomas were ZN positive with mild to moderate numbers of acid-fast bacilli in the necrotic centres and in macrophages (Figure 2).

Aeromonas hydrophila was isolated and identified from the kidney. Parasitological investigations were negative.

From all samples it was possible to isolate non-chromogenic colonies on the two media and the purified isolates were identified by phenotypical and biochemical characterisation techniques as *M. fortuitum* in both the spleen and the liver. BLAST analysis identified both strains as *M. fortuitum* with a 100% identity rate (E-value 0.0) with the reference strain DSM 46621 (ATCC 6841). The sequence has been submitted to

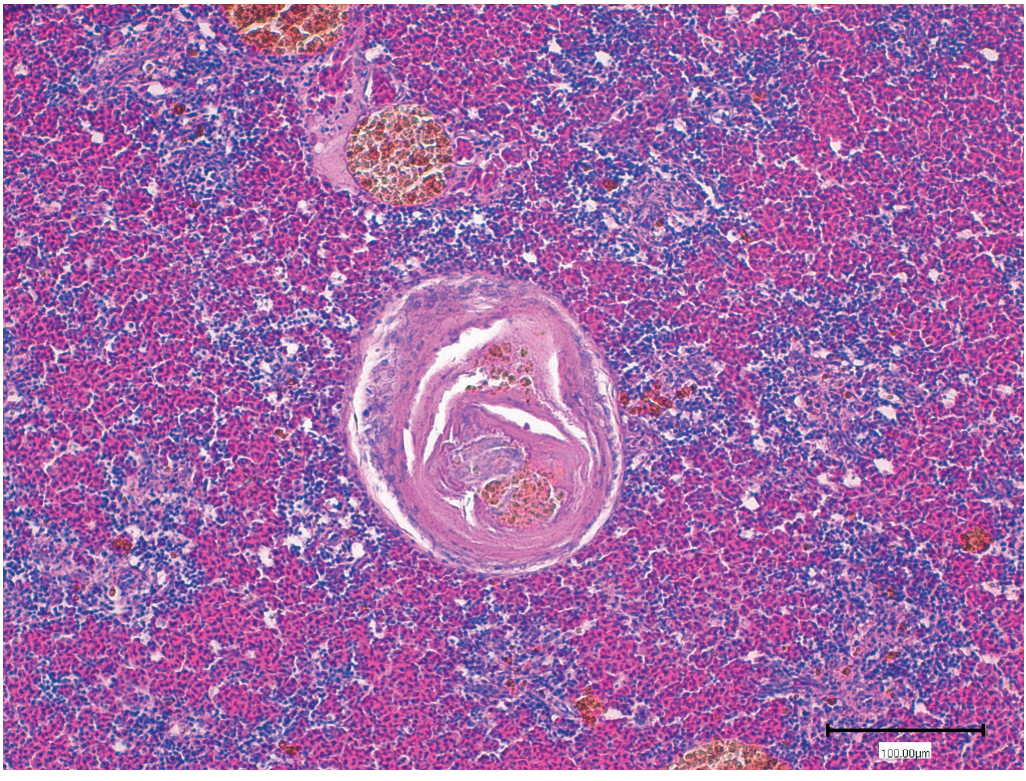

Figure 1. Spleen: granulomatous lesion with a central area of eosinophilic cellular debris with dark brown pigment surrounded by inflammatory cells and enclosed by a thick capsule (HE) bar = 100µm.

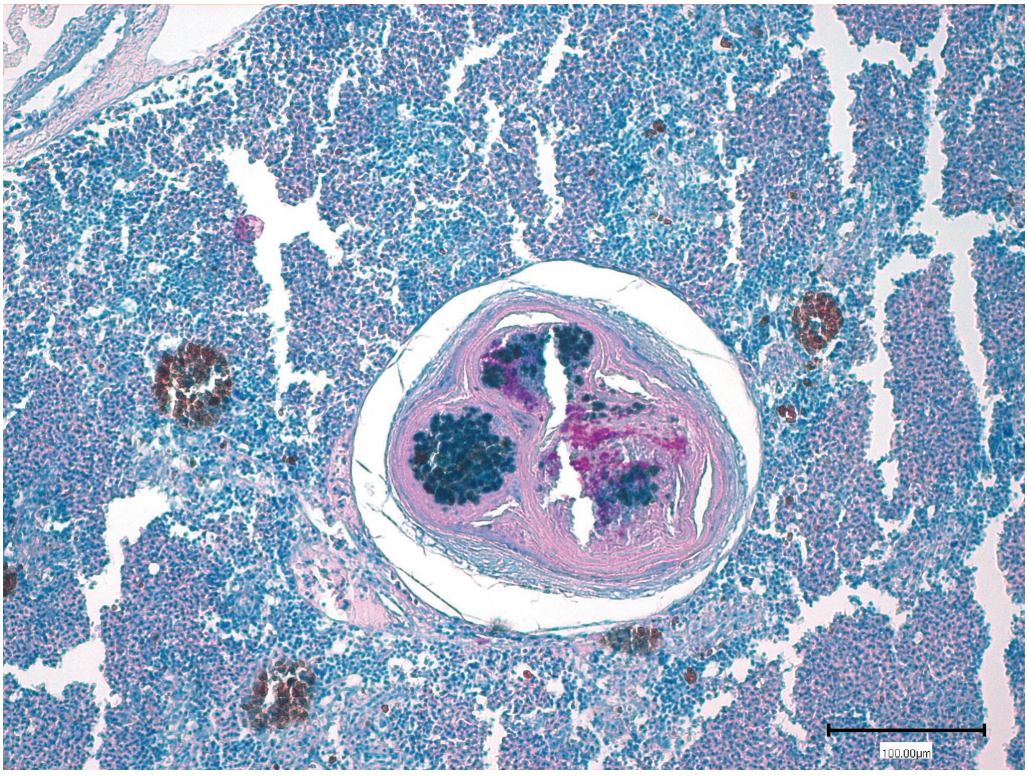

Figure 2. Spleen: acid-fast bacilli within the necrotic core of the granuloma (ZN) bar = 100µm.

GenBank with the accession number MF280110. To our knowledge this is the first report of mycobacterial infection in this fish species caused by *M. fortuitum*.

On the basis of the distribution and characteristics of the microscopical lesions found in the subject, we can hypothesise that the mycobacterial infection should not to be considered as the primary cause of death of the fish. Indeed, the presence of multifocal microscopic lesions at an advanced stage of evolution indicates that this is a chronic infection. The cause of death could be referred to a multifactorial complex of events linked to aquarium management mistakes, also confirmed by the circulation of *A. hydrophila*, a bacterium characterised by low pathogenicity, but reported as the cause of systemic facultative bacterial infections related to poor environmental conditions (Noga, 2010).

The case described, characterised by the presence of a chronic infection in a subject without symptoms, reiterates that the zoonotic risk for aquarists and sellers of ornamental fish is real. In this regard, the correct management of the aquarium is therefore of fundamental importance in order to prevent potential health problems for the professional figures involved and it can be achieved by complementing correct

sanitization and disinfection practices along with control of the batches of imported fish (Decostere et al., 2004).

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence the content of the paper.

References

Allen GR, Midgley SH and Allen M (2002). In "**Field guide to the freshwater fishes of Australia**", pp. 56-58. Perth, Western Australia Museum.

Decostere A, Hermans K and Haesebrouck F (2004). Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. *Veterinary Microbiology* **99 (3-4)**, 159-66.

Garcia A, Tello S, Vargas G and Duponchelle F (2009). Patterns of commercial fish landings in the Loreto region (Peruvian Amazon) between 1984 and 2006. *Fish physiology and biochemistry* **35**, 53–67.

Gauthier DT and Rhodes MW (2009). Mycobacteriosis in fishes: A review. *The Veterinary Journal* **180 (1)**, 33-47.

Heckert RA, Elankumaran S, Milani A and Baya A (2001). Detection of a new mycobacterium species in wild striped bass in the Chesapeake Bay. *Journal of Clinical Microbiology* **39**, 710-715.

Kent PT and Kubica GP (1985). "**Public Health Mycobacteriology. A Guide for the Level III Laboratory**". US Department of Health and Human Services, Centers for Disease Control, Atlanta, GA.

Lee WJ, Kang SM, Sung H, Won CH, Chang SE, Lee MW, Kim MN, Choi JH and Moon KC (2010). Non-tuberculous mycobacterial infections of the skin: a retrospective study of 29 cases. *Journal of Dermatology* **37 (11)**, 965-972.

Maldonado-Ocampo JA, Vari RP and Usma JS (2008). Checklist of the freshwater fishes of Colombia. *Biota Colombiana* **9 (2)**, 143-237.

Noga EJ (2010). Motile Aeromonas Infection (MAI; Motile *Aeromonas* Septicemia [MAS] Red Sore). In "**Fish Disease Diagnosis and Treatment**", pp. 185-186. Wiley-Blackwell. ISBN: 978-0-813-80697-6.

Puk K, Banach T, Wawrzyniak A, Adaszek Ł, Ziętek J, Winiarczyk S and Guz L (2018). Detection of *Mycobacterium marinum*, *M. peregrinum*, *M. fortuitum* and *M. abscessus* in aquarium fish. *Journal of Fish Diseases* **41 (1)**, 153-156.

Rhodes MW, Kator H, Kaattari I, Gauthier D, Vogelbein W and Ottinger CA (2004). Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. *Diseases of Aquatic Organisms* **61**, 41-51.

Rhodes MW, Kator H, McNabb A, Deshayes C, Reyrat JM, Brown-Elliott BA, Wallace R, Trott KA, Parker JM, Lifland BD, Osterhout G, Kaattari I, Reece K, Vogelbein WK and Ottinger CA (2005). *Mycobacterium pseudoshottsii* sp. *nov.*, a slowly growing chromogenic species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). *International Journal of Systematic and Evolutionary Microbiology* **55**, 1139-1147.

Righetti M, Favaro L, Antuofermo E, Caffara M, Nuvoli S, Scanzio T and Prearo M (2014). *Mycobacterium salmoniphilum* infection in a farmed Russian sturgeon, *Acipenser gueldenstaedtii* (Brandt & Ratzeburg). *Journal of Fish Diseases* **37 (7)**, 671-674.

Telenti A, Marchesi F, Balz M, Bally F, Böttger EC and Bodmer T (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *Journal of clinical microbiology* **31**, 175-178.

Tovar Verba J, Rabello Neto JG, Zuanon J and Farias I (2014). Evidence of multiple paternity and cooperative parental care in the so called monogamous silver

arowana *Osteoglossum bicirrhosum* (Osteoglossiformes: Osteoglossidae). *Neotropical Ichthyology* **12 (1)**, 145-151.

Wayne LG and Kubica GP (1986). The mycobacteria. In "**Bergey's Manual of Systematic Bacteriology**" (P.H.A. Sneath, N.S. Mair, M.F. Sharpe & J.G. Holt, Ed.), pp. 1435–1457. Williams & Wilkins, Baltimore, MD.

Zanoni RG, Florio D, Fioravanti ML, Rossi M and Prearo M (2008). Occurrence of *Mycobacterium* spp. in ornamental fish in Italy. *Journal of Fish Diseases* **31 (6)**, 433-41.