

Postmortem isolation of *Mycobacterium marinum* from yellow-bellied slider (*Trachemys scripta scripta*)

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Summary

Mycobacterium marinum, a slow-growing non-tuberculous mycobacterium, is known for causing skin infections in fish and amphibians, commonly known as “fish tank granuloma” or “swimming pool granuloma”. While its primary impact is on aquatic species, humans also face a potential risk through exposure to contaminated water or aquatic environments. In humans, infections typically are manifested as nodular skin lesions, often on the hands or fingers, leading to a chronic and indolent course. Due to its unique ability to thrive in aquatic environments, *M. marinum* serves as an infectious agent not only for fish but also for other animal species, including reptiles, especially turtles. The turtle case of postmortem isolation of *M. marinum* described in this work serves as a reminder of the threat posed by this bacterium, highlighting the relatively low awareness surrounding this subject not only among owners but also among veterinarians. Expanding our understanding of the threats posed by *M. marinum* is crucial, not only for the well-being of aquatic animals, including turtles, but also for human health. Given the zoonotic potential of *M. marinum*, a comprehensive understanding of its transmission dynamics, clinical manifestations, and preventive measures is essential. Hence this knowledge can inform strategies to minimize the risk of *M. marinum* infections overall.

Keywords: *Mycobacterium marinum*, turtle, reptiles, fish, PCR

Mycobacterium marinum is atypical mycobacteria that is pleomorphic, Gram-positive, aerobic, nonmotile, acid-fast bacillus, and belongs to Group I of Runyon's classification. *M. marinum* can invade macrophages in a similar way as other mycobacterium, preventing phagosome-lysosome fusion and replicating inside them. The organism can survive, replicate in macrophages, and even escape from the phagosomes into the cytoplasm, where it can induce actin polymerisation leading to direct cell spread (7). *M. marinum* is a key fish pathogen, associated with multiple symptoms, e.g. uncoordinated swimming, abdominal swelling, weight loss, skin ulceration, white nodule formation in the various organs such as liver, kidney, spleen in both fresh and marine fish (6). The fish model of *M. marinum* has shown that it may occur in two different courses. Acute disease is characterised

by uncontrolled growth of the pathogen and the death of all animals in two days, while chronic disease is characterised by the formation of granulomas in different organs and the survival of animals for at least 4 to 8 weeks (19).

Mycobacteriosis caused by *M. marinum* has been detected in several animal species except fish including bearded dragon (*Pogona vitticeps*) (9) and pygmy hedgehog (*Atelerix albiventris*) (2). The clinical symptoms of the mentioned species are quite similar and include: emaciation, apathy, swelling of joints and limbs, and associated lameness, difficult-to-heal warty, pustular, exfoliating ulcers of the skin, epidermis, and its products such as nails, scales, beak horn sheath, or spines. The anatomopathological changes of chronic general infection include granulomatous changes in organs, mainly the liver, kidneys, spleen, lungs, and lymph nodes, and enlargement of the affected organs.

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To the best of our knowledge, the literature on death cases of turtles caused by *M. marinum* is limited (15). So far, the occurrence of *M. fortuitum*, *M. chelonae*, *M. avium* ssp. *avium*, *M. nonchromogenicum*, *M. neoaurum* and *M. scrofulaceum* has been reported among invasive turtles in Poland but there is no data in the available literature on the isolation of *M. marinum* (17). The aim of this study was to describe a fatal case of a turtle infected with *M. marinum*, with the goal of raising awareness among pet owners and veterinarians about the risks posed by *M. marinum* in aquaculture and its zoonotic potential.

Material and methods

Patient. The patient, a 3-year-old yellow-bellied slider (*Trachemys scripta troostii*), experienced cardiac arrest and had been previously treated at another veterinarian's office. The turtle's medical history revealed concerning symptoms, including a significant weight loss (from 400 g to 270 g within a month), reduced appetite, lethargy, diminished responsiveness to stimuli, yellowing of the skin around the abdomen, pustules on the limb palms, peeling skin on the limbs, and shedding of carapace horn plates (notably evident during submersion, with hanging patches). Despite the carapace retaining firmness under pressure, there was localized damage. The turtle exhibited intense nasal discharge, slight tissue swelling around the eyes, and an extended duration spent under the heat lamp. Notably, during swimming, a subtle sinking of the right side of the body was observed. An initial diagnosis by a previous veterinarian suspected pneumonia and skin mycosis, leading to a prescription of fungiderm (clotrimazolum 0.005 g/ml, Biowet Drwalew, Poland), tulathromycin, meloxicam, immunostimulating drugs, and vitamins A, E, and D₃. However, a follow-up visit after a month revealed no significant improvement in the turtle's health. Subsequently, the antibiotic regimen was altered to lincomycin with spectinomycin, along with ornipural and duphalyte. Additional recommendations included 15-minute potassium permanganate baths, iodine application to lesions, use of Fungiderm solution, and the application of olive oil to the carapace. Despite these interventions, no improvement was observed, and the turtle's condition deteriorated approximately one week after the completion of antibiotic therapy, marked by limb swelling, skin ulcers, and claw loss. Unfortunately, a few days later, the turtle succumbed to the illness.

He was fed once a day: frozen and dried shrimps, dried baby shrimp, frozen food for turtles in blisters, dried gammarus, cryptocoryne, biorept food-feed mixture for turtles (Tropical, Poland), aquarium fish (live, dead) occasionally. Maintained under controlled conditions in a 25-degree Celsius heater with UVB lamp 3 months before his death.

The autopsy performed in the day of death revealed notable skin alterations, along with minor and major nodules in the liver and spleen (Fig. 1A). Based on these findings and the medical history, a diagnosis of mycobacteriosis was established, as one probability. Furthermore, an examination of the fish used as a food source for the turtle, revealed multiple individuals displaying clinical signs concerning for mycobacterial infection, including apathy, morphologi-

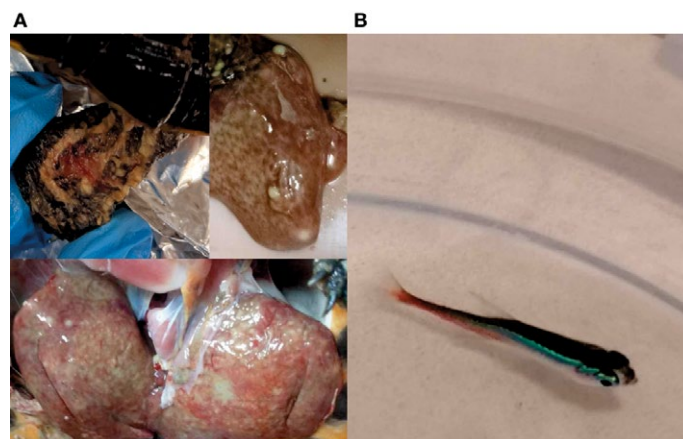


Fig. 1. Lesions on the feet and minor and major lumps in the liver (A). The neon tetra (*Paracheirodon innesi*) with shape changes common during *M. marinum* infection (B)

cal changes, and sudden fatal failure (Fig. 1B). Histology was not pursued due to the delayed delivery of turtle to the veterinarian post-mortem and autolysis. Liver impression cytology was performed, and a fragment of liver and spleen was taken in 70% ethanol for molecular analysis.

Ziehl-Neelsen staining. All cytology preparations were stained by the conventional Ziehl-Neelsen method for the presence of acid-fast bacilli (AFB) as depicted earlier (13) and observed under a light microscope.

DNA isolation. The tissue fragments collected during the turtle autopsy were fixed in 70% ethanol and transported to the Animallab Veterinary Laboratory in Warsaw. Tissues with visible lumpy lesions were first hydrated with a 0.9% saline solution overnight. Additionally, they were manually homogenized and suspended in 0.3 mL of deionized water. The manual DNA extraction was performed according to the manufacturer's instructions with the addition of 20 µl of 0.1 M dithiothreitol during the first step of isolation (Sherlock AX, A&A Biotechnology). The specimen was incubated at 50°C until it was all lysed. The obtained DNA was frozen for further tests.

PCR and sequencing. A sample was tested by PCR protocol targeting a 614-bp fragment of 16S rRNA of *Mycobacterium marinum*. PCR was performed in a 50 µl reaction mixture containing 25 µl of StartWarm HS-PCR Mix (A&A Biotechnology) and 3 µl each primer: 246F 5'-AGAGTTT-GATCCTGGCTCAG-3' and 266R 5'-CACGCYCACAGT-TAAGCYGT-3', 14 µl dd H₂O, and 5 µl of template DNA. Primers used in our study were designed by Böddinghaus et al. (3). PCR was carried out by an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min in a PCR thermocycler (MultiGene optiMAX, Labnet International Inc.) (20). Amplified products were analyzed on a 2% agarose gel. The PCR product was sequenced and then aligned, analyzed with MEGA 11 (Molecular Evolutionary Genetics Analysis Version 11), and compared with the GenBank® database.

Results and discussion

Currently, mycobacterial culture is the gold standard for detecting *Mycobacterium* spp. but it is time-con-

suming and requires specialized safety procedures in laboratories (16). In our case where the cadaver was delivered late, conducting mycobacterial culture had a high risk of failure; thus, we chose staining the immersion cytology from the liver. Ziehl-Neelsen staining reveals large quantities AFB in liver immersion cytology (Fig. 2).

Conventional microscopy with Ziehl-Neelsen (ZN) staining is a rapid and practical method for detecting AFB, especially in low-income countries, due to its rapidity, low cost, and high positive predictive value for mycobacteriosis (13). The PCR technique is a rapid and sensitive method that is becoming more and more popular in mycobacterium diagnostics (14). In this case, we use the simplest method to confirm presence of AFB by ZN and at the same time the most sensitive and specific method, i.e. PCR defining a specific species *M. marinum* which we additionally extended by sequencing the bacterium genome causing the infection. Electrophoresis reveal the 614-bp PCR product isolated from turtle tissues (Fig. 3). Then it was used for sequencing.

Dideoxy-termination sequencing (Sanger sequencing) was performed in a commercial laboratory. Sequencing was repeated twice thus an unreadable chromatograph. Complete alignment was achieved using two strands for both primers (246F and 266R). Only the 509-bp fragment of the amplified gene had a completely compatible alignment for both strands.

The obtained 509-bp product was 100% homologous to *Mycobacterium marinum* sequences coding 16S rRNA present in GenBank® (e.g., MN450809, AF456239, and MG009242). The obtained sequence has been submitted as OR842238.

The prolonged treatment of the turtle without improvement underscores the insidious nature of *M. marinum*, as it can masquerade as various diseases. This challenge is not limited to veterinary medicine alone but extends to human medicine (8), emphasizing the heightened importance of raising awareness about this disease.

Depending on the isolate of *M. marinum* isolate, the fish developed acute or chronic disease. Acute disease was characterised by uncontrolled growth of the pathogen and death of all animals in 16 days, whereas chronic disease was characterised by the formation of granulomas in different organs and survival of the animals for at least 4 to 8 weeks (19).

As it was mentioned, mycobacteriosis, especially *M. marinum*, may also concern other species, while other aquatic vertebrates may be a source of infection to fishes. Moreover, invertebrate organisms such as snails

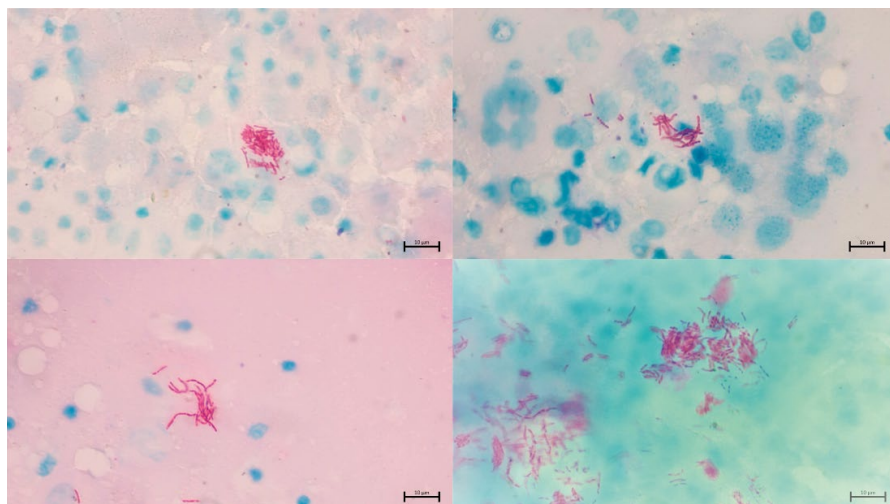


Fig. 2. Acid-fast bacilli present in large quantities in liver immersion cytology
 Explanations: Ziehl-Neelsen staining, $\times 1000$, bar = 10 μm

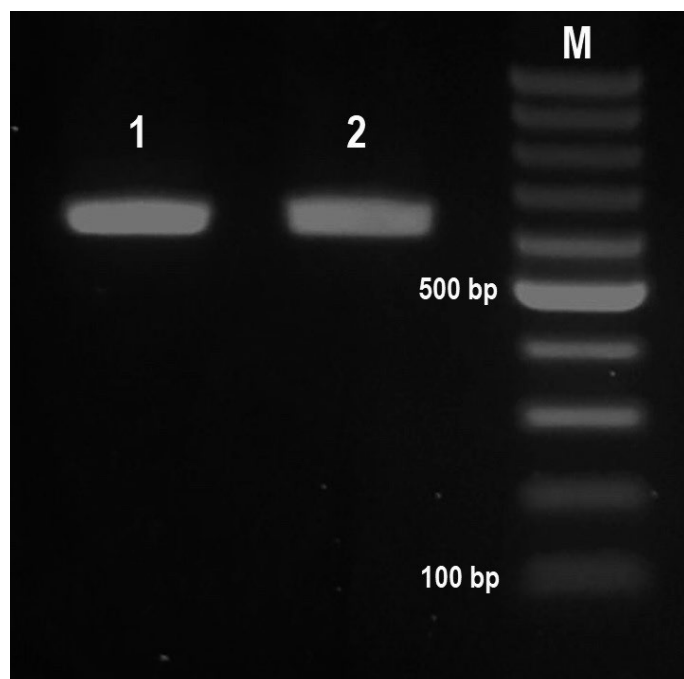


Fig. 3. The 614-bp PCR products of 16S rRNA of *M. marinum*
 Explanations: lanes 1 and 2 – isolated turtle tissues; lane M – DNA Ladder Marker 1 (A&A; A Biotechnology)

and water fleas play a crucial role in the transmission of this agent (18).

Transmission between fish occurs by ingestion of infected fish, by contact with skin lesions, or through the gills. In the previously mentioned vertebral case, *M. marinum* confirmed that the route of transmission was oral because a bearded dragon was fed infected guppies approximately two weeks before the onset of symptoms (5). This route is also very possible in case of the turtle described in this article. But we cannot also exclude transmission by contaminated equipment used even for the cleaning of tanks. Such a transmission route was postulated in the case of *M. marinum* in blue-fronted amazon parrot where infection occurs after incorrect beak trim where the surgical equipment might have been contaminated (10).

According to treatment guidelines among the drugs acting on *M. marinum*, the possible treatment includes macrolides and aminoglycosides. However, antimicrobial treatment is not able to eliminate *Mycobacterium* spp. from affected fish colonies; the infection is only completely controlled after culling and disinfection of infected stocks (21). Similarly, as in the case of other species, there is no effective treatment.

This case underscores the underdiagnosed nature of *M. marinum* infections and sheds light on the limited awareness among fish owners regarding the threat it poses, not only to various species but also to humans. In mammals, including humans, infections are commonly linked to damaged skin exposure to contaminated water from fish basins or affected objects (11). *Mycobacterium marinum* is one of the causes of extrapulmonary mycobacterial infections, which in humans is typically manifested as simple skin lesions limited to the skin and soft tissues in immunocompetent patients, while disseminated forms may occur in immunocompromised hosts, such as those with HIV/AIDS (1). Although infections can result from direct injuries caused by fish fins or bites, the majority are typically acquired during aquarium maintenance tasks, such as cleaning or water changes (12). Treatment typically involves the administration of two active antimycobacteriosis drugs, guided by microbial susceptibility testing in culture, where macrolides and rifampin play pivotal roles. In some cases, surgery may also be considered (4). This highlights the importance of comprehensive awareness and preventive measures in mitigating the risks associated with *M. marinum*, both for the well-being of aquatic species and for safeguarding human health.

This work marks another documented case of post-mortem isolation of *M. marinum* in a turtle, which underscores the potential threat *M. marinum* poses not only to turtles but also to various species that are not kept or fed. Consequently, public awareness of the hazards that fish may pose to both humans and their animals remains alarmingly inadequate.

Typically, *M. marinum* infections in fish and aquarium animals have a poor prognosis, often necessitating symptomatic treatment with euthanasia as a possible recommendation if the condition deteriorates. It is imperative to educate owners of aquatic animals but also veterinarians about the risks associated with *M. marinum*.

Furthermore, given the zoonotic potential of this mycobacterium, it is crucial to highlight the substantial risk it poses to humans. Beyond the documented cases, emphasizing the importance of proper hygiene, handling practices, and preventive measures is paramount in mitigating the potential transmission of *M. marinum* from aquatic environments to humans. Increasing awareness among owners, veterinarians, and the general public is essential to restrain the spread of infections and promote a safer environment for both animals and humans.

References

1. Aubry A., Mougari F., Reibel F., Cambau E. (ed.): *Mycobacterium marinum* infection. Schlossberg D. L.: Tuberculosis and Nontuberculous Mycobacterial Infections, 7th edn. John Wiley & Sons, New Jersey 2017, p. 735-752, doi: 10.1128/9781555819866.ch43.
2. Blume G. R., Eloi R. S. A., Oliveira L. B., Moraes E. L. S. C., Seeger M. G., Cargnelutti J. F., de Sant'Ana F. J. F.: Non-tuberculous Mycobacterial Granulomatous Dermatitis in an African Pygmy Hedgehog (*Atelerix albiventris*). J. Comp. Pathol. 2021, 182, 22-26, doi: 10.1016/J.JCPA.2020.11.003.
3. Boddingtonhaus B., Rogall T., Flohr T., Blocker H., Bottger E. C.: Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. 1990, 2, 1751, doi: 10.1128/JCM.28.8.1751-1759.1990.
4. Canetti D., Riccardi N., Antonello R. M., Nozza S., Sotgiu G.: Mycobacterium marinum: A brief update for clinical purposes. Eur. J. Intern. Med. 2021, 105, 15-19, doi: 10.1016/J.EJIM.2022.07.013.
5. Delghandi M. R., El-Matbouli M., Menanteau-Ledouble S.: Mycobacteriosis and infections with non-tuberculous mycobacteria in aquatic organisms: a review. Microorganisms 2020, 8, 1-18, doi: 10.3390/MICROORGANISMS8091368.
6. El Amrani M. H., Adoui M., Patey O., Asselineau A.: Upper extremity Mycobacterium marinum infection. Orthop. Traumatol. Surg. Res. 2010, 96, 706-711, doi: 10.1016/J.OTSR.2010.02.016.
7. El-Etr S. H., Yan L., Cirillo J. D.: Fish monocytes as a model for mycobacterial host-pathogen interactions. Infect. Immun. 2001, 69, 7310-7317, doi: 10.1128/IAI.69.12.7310-7317.2001.
8. Fata A., Bojdy A., Maleki M., Faras B. R. H., Ghazvini K., Tajzadeh P., Vakili V., Moghaddas E., Mastroeni P., Rahmani S.: Fish tank granuloma: An emerging skin disease in Iran mimicking Cutaneous Leishmaniasis. PLoS One. 2019, 14, doi: 10.1371/JOURNAL.PONE.0221367.
9. Girling S. J., Fraser M. A.: Systemic mycobacteriosis in an inland bearded dragon (*Pogona vitticeps*). Vet. Record. 2007, 160, 526-528, doi: 10.1136/VR.160.15.526.
10. Hannon D. E., Bemis D. A., Garner M. M.: Mycobacterium marinum infection in a blue-fronted Amazon parrot (*Amazona aestiva*). J. Avian Med. Surg. 2012, 26, 239-247, doi: 10.1647/2011-058.
11. Hashish E., Merwada A., Elgaml S., Amer A., Kamal H., Elsadek A., Marei A., Sitohy M.: Mycobacterium marinum infection in fish and man: epidemiology, pathophysiology and management; a review. Vet. Q. 2018, 38, 35-46, doi: 10.1080/01652176.2018.1447171.
12. Jernigan J. A., Farr B. M.: Incubation period and sources of exposure for cutaneous Mycobacterium marinum infection: case report and review of the literature. Clin. Infect. Dis. 2000, 31, 439-443, doi: 10.1086/313972.
13. Koch M. L., Cote R. A.: Comparison of fluorescence microscopy with Ziehl-Neelsen stain for demonstration of acid-fast bacilli in smear preparations and tissue sections. Am. Rev. Respir. Dis. 1965, 91, 283-284, doi: 10.1164/ARRD.1965.91.2.283.
14. Lin C. R., Wang H. Y., Lin T. W., Lu J. J., Hsieh J. C. H., Wu M. H.: Development of a two-step nucleic acid amplification test for accurate diagnosis of the Mycobacterium tuberculosis complex. Sci. Rep. 2021, 11, 1-11, doi: 10.1038/s41598-021-85160-2.
15. Merola C. T., Hauber L., Graham E. A., Ossiboff R., DiGeronimo P. M.: Pathology in Practice. J. Am. Vet. Med. Assoc. 2022, 259, 1-4, doi: 10.2460/JAVMA.20.06.0323.
16. Mishra A., Singhal A., Chauhan D. S., Katoch V. M., Srivastava K., Thakral S. S., Bharadwaj S. S., Sreenivas V., Prasad H. K.: Direct detection and identification of Mycobacterium tuberculosis and Mycobacterium bovis in bovine samples by a novel nested PCR assay: Correlation with conventional techniques. J. Clin. Microbiol. 2005, 43, 5670-5678, doi: 10.1128/JCM.43.11.5670-5678.2005/ASSET/115A524D-FB4A-4D24-A4BC-C967D530FAC9/ASSETS/GRAPHIC/ZJM0110560090002.JPEG.
17. Radulski L., Krajewska-Wędzina M., Lipiec M., Weiner M., Zabost A., Augustynowicz-Kopeć E.: Mycobacterial infections in invasive turtle species in Poland. Pathogens 2023, 7, 12, 4, 570, doi: 10.3390/pathogens12040570.
18. Rovid Spickler A., Dvorak G.: Piscine Mycobacteriosis 2006.
19. Sar A. M., Van Der, Abdallah A. M., Sparrus M., Reinders E., Vandenbroucke-Grauls C. M. J. E., Bitter W.: 2004. Mycobacterium marinum strains can be divided into two distinct types based on genetic diversity and virulence. Infect. Immun. 2002, 72, 6306-6312, doi: 10.1128/IAI.72.11.6306-6312.2004.
20. Ucko M., Colorni A., Kvitt H., Diamant A., Zlotkin A., Knibb W. R.: Strain variation in Mycobacterium marinum fish isolates. Appl. Environ. Microbiol. 2002, 68, 5281-5287, doi: 10.1128/AEM.68.11.5281-5287.2002.
21. Yanong R. P. E., Pouder D. B., Falkinham J. O.: Association of mycobacteria in recirculating aquaculture systems and mycobacterial disease in fish. J. Aquat. Anim. Health. 2010, 22, 219-223, doi: 10.1577/H10-009.1.

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