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Occurrence and antimicrobial susceptibility of *Mycobacterium peregrinum* in ornamental fish

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Abstract

Systemic mycobacteriosis was diagnosed in a group of ornamental fish. Although a large number of acid-fast bacterial rods were identified in the kidneys, liver, and muscles of each fish, no granulomas were observed in internal organs. *Mycobacterium peregrinum* was identified using the GenoType Mycobacterium CM assay. This study illustrates a considerable risk of atypical mycobacteriosis in humans.

Key words: ornamental fish, *Mycobacterium*, genotyping, drug resistance.

Introduction

Mycobacteria are a group of microorganisms that can be isolated from a wide range of environmental conditions. Over 130 non-tuberculous mycobacteria have been identified, and approximately one third have been associated with diseases in humans. Most species are free-living and ubiquitous in the environment and have been found in soil and various aquatic sources, including municipal tap water and hospital water systems. *Mycobacterium fortuitum* complex (*M. fortuitum*, *M. peregrinum* type 1, and *M. peregrinum* type 2), *M. avium* complex (*M. avium*, *M. intracellulare*), *M. chelonae-M. abscessus* complex (*M. abscessus* and *M. chelonae*), *M. scrofulaceum*, *M. kansasii,* and *M. marinum* are common in the environment (9, 10, 11, 17). It is increasingly recognised that humans may be infected or colonised with non-tuberculous mycobacteria, "atypical mycobacteria" or "mycobacteria other than *M. tuberculosis*" (MOTT) (24, 31).

The first case of mycobacteriosis in freshwater fish was described by Bataillon *et al.* (4) in the common carp (*Cyprinus carpio*). Fish mycobacteriosis is well-recognised in marine and freshwater fish, with several reviews concerning the topic (6, 7, 12). Mycobacterial infections are one of the most common infections of ornamental fish. Many mycobacterial species have been isolated from fish tissues. The following species have been detected: *M. abscessus*, *M. aurum*, *M. avium* complex, *M. chelonae*, *M. chesopeaki*, *M. fortuitum*, *M. gordonae*, *M. haemophilum*, *M. insubricum, M. interjectum*, *M. kansasii*, *M. marinum, M. montefiorense*, *M. peregrinum, M. parafortuitum, M. poriferae, M. pseudoshottsii*, *M. scrofulaceum*, *M. shottsii*, *M. similae*, *M. stomatepiae* sp. nov., *M. szulgai*, and *M. triplex* (1, 7, 13, 32). Environmental mycobacteria can be pathogenic to a variety of freshwater and marine fish species both in the wild and kept in captivity (15, 21). No fish species should be considered immune. Barbs (*Barbus tetrazoma*), bettas (*Betta splendens*), cichlids (*Apistogramma cacatuoides*), danios (*Danio rerio*), discus fish (*Symphysodon aequifasciatus*), goldfish (*Carassius auratus auratus*), gouramis (*Trichogaster trichopterus*, *Colisia lalia*), oscars (*Astronotus ocellatus*), platyfish (*Platypoecillus maculates*), red-bellied piranha (*Pygocentrus nattereri*), and poeciliids (*Poecilia mexicana*, *Xiphophorus hellerii*) may be particularly susceptible

(3, 13, 20, 22, 29). Clinical signs of fish mycobacteriosis are quite variable and usually include loss of appetite, lethargy, staying alone in the corner of the aquarium, floating on the surface of the water, anorexia, skin discoloration, open lesions, ulcerations, unilateral or bilateral exophthalmia, emaciation, skeletal deformities, and well-demarcated granulomas in internal organs (7, 25). Mycobacteria were also isolated from skin, gills, musculature, and internal organs of clinically healthy fish (5, 33).

This study describes the infection caused by *Mycobacterium peregrinum* in healthy ornamental fish.

Material and Methods

Fish and gross examination. A total of 12 apparently healthy fish were used, which originated from the aquarium of a fish breeder who suffered from a *Mycobacterium*-like skin infection. Black mollies (*Poecilia sphenops*), guppies (*Poecilia reticulata*), green swordtails (*Xiphophorus hellerii*), zebra fish (*Danio rerio*), and pangasius catfish (*Pangasius hypophthalmus*) were examined.

Fish without clinical signs of the disease were euthanised with 200 mg L^{-1} tricaine methane sulfonate (MS-222) (Syndel, Canada) (6). Imprints of the kidneys, liver and muscles were stained with Ziehl-Neelsen (Merck, Darmstadt, Germany). Bacteria were isolated by the streaking method using an agar slant of Petragnani egg medium (PIWet/PIB, Poland) and another of Stonebrink egg medium (PIWet/PIB, Poland) (20). The tissues were homogenised, and suspensions were mixed with an equal volume of 5% oxalic acid solution (PIWet/PIB, Poland) and incubated for 15 min. Afterwards, the samples were centrifuged at 3000 g for 15 min at 4°C. The pellets were washed twice in sterile PBS (Gibco Invitrogen, New Zealand), and cultured on egg media at 26°C for 6 weeks. Colonies that were positive after Ziehl-Neelsen staining were subcultured at 26°C and 37°C for one week, using two agar slants.

GenoType CM assay. The GenoType Mycobacterium CM (Hain Lifescience, Germany) assay was performed as recommended by the manufacturer. DNA isolations and amplification reactions were performed according to the manufacturer's instructions (GenoType system - Hain Lifescience, Germany). Hybridisation and detection were carried out in an automated system (TwinCubator, Hain Lifescience, Germany) according to the manufacturer's instructions: 20 µL of the amplification product was incubated for 5 min at room temperature with 20 µL of denaturation reagent. Then 1 mL of hybridisation buffer was added per well, and the membrane strips were placed into each well. The hybridisation was performed at 45°C for 30 min. After washing in 1 mL of stringent wash solution at 45°C for 15 min and then in 1 mL of rinse solution at room temperature for 1 min, the strips were incubated for 30 min at room temperature with 1 mL of alkaline phosphatase conjugated with streptavidin. The strips were washed three times at room temperature: twice in 1 mL of rinse solution for 1 min and once in 1 mL of water for 1 min. They were then incubated in 1 mL of the substrate at room temperature, in darkness, for 3-20 min. The reaction was stopped by washing the strips twice with water. After drying the strips, the pattern of stained bands corresponding to specific DNA probes was evaluated by comparison with the model provided by the manufacturer. The assessment only concerned strips with well-stained control bands.

Drug susceptibility tests. Drug susceptibility was tested using the 1% proportion method in Löwenstein-Jensen medium. The following drugs were tested: amikacin sulfate (Bioton, Poland), streptomycin (Polfa Tarchomin, Poland), isoniazid (Sigma Chemicals, USA), clofazimine (Sigma Chemicals, USA), capromycin sulfate (Eli Lilly & Co., USA), ofloxacin (Sigma Chemicals, USA), ethionamide (Sigma Chemicals, USA), ethambutol (Teva Pharmaceuticals, Poland), ryfampicin (Sigma Chemicals, USA), Biseptol [co-trimoxazolum (sulfamethoxazolum + trimethoprimum)] (Polfa Tarchomin, Poland), Davercin (erythromycin cyclocarbonate) (Polfa Tarchomin, Poland), and rifabutin (Sigma Chemicals, USA).

Results

A *Mycobacterium*-like infection was present on the skin of an aquarium fish breeder who cut himself on glass from an aquarium containing ornamental fish. Several weeks after the cut healed, thick, reddish knots appeared that seemed to spread and grow. Some unsuccessful attempts at treatment were made (data not shown).

Among the 12 fish examined, no clear macroscopic findings of tuberculosis *i.e.* emaciation, inflammation of the skin, exophthalmia ("pop-eye"), oedema, skin ulceration, keratitis, skeletal deformities, or visceral nodules were observed. Neither were granulomas detected in any fish after macroscopic examination. The fish were found to be positive for mycobacteria in bacteriological examination, and acid-fast rods were observed in imprint preparations of the liver, kidneys, and muscles (Table 1). The isolates were analysed with the GenoType *Mycobacterium* CM assays (for common mycobacteria). Using this test, *M. peregrinum* was identified in apparently healthy fish (Fig. 1).

The bactericidal activity of particular antibiotics is presented in Table 2. Out of the 12 antibiotics tested only five showed bactericidal activity against the isolates, and out of them the three comprising ofloxacin, capromycin, and amikacin were active against all isolates.

Table 1. Results of identification of mycobacterial isolates in ornamental freshwater fish

Fish species	Number of fish	Muscle, liver, kidney			
		P[a]	C[b]	ZN[c]	GenoType CM assay
Poecilia sphenops	3	-	+	+	*M. peregrinum*
Poecilia reticulata	2	-	+	+	*M. peregrinum*
Xiphophorus hellerii	3	-	+	+	*M. peregrinum*
Danio rerio	2	-	+	+	*M. peregrinum*
Pangasius hypophthalmus	2	-	+	+	*M. peregrinum*

[a] Detection of granulomas by pathological (P) examination
C[b] - Culture examination
ZN[c] Ziehl-Neelsen staining; - acid-fast rods not detected; + acid-fast rods detected

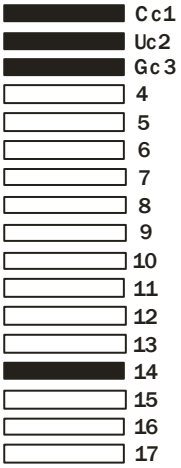


Fig. 1. Identification of *M. peregrinum* by the use of the GenoType CM assay. The lines (from top to bottom) are as follows: 1 - conjugate control (Cc); 2 - universal control (Uc); 3 - genus control (Gc); 4-17 - specific probes for the identification of mycobacterial species.

Table 2. Antimicrobial drug sensitivity of *Mycobacterium peregrinum* isolates measured by the proportion method (n = 12) on Löwenstein-Jensen agar

Antibiotics	Drug concentration (mg/L)	Resistance (%)
Streptomycin	4.0	100
Isoniazid	0.2	100
Ofloxacin	2.5	0
Ethionamide	40.0	33.3
Ethambutol	2.0	100
Capreomycin	40.0	0
Ryfampicin	40.0	100
Amikacin	0.016	0
Biseptol[a]	16	100
Clofazimine	2	100
Davercin[b]	12	100
Rifabutin	2	33.3

[a] Biseptol [co-trimoxazolum (sulfamethoxazolum + trimethoprimum)]
[b] Davercin (erythromycin cyclocarbonate)

Discussion

Fish mycobacteriosis, caused by *M. peregrinum*, has been previously reported in cultured Pacific white shrimp (*Penaeus vannamei*) (18) and in fish (2, 13, 22). A very rare case of *M. peregrinum* infection in healthy freshwater aquarium fish was reported. Usually, fish mycobacteriosis causes chronic to subacute disease, and most infected fish do not normally exhibit any symptoms, but when they are stressed they die in large numbers, and granulomas are often seen in infected tissues (25). In this study, in fish which revealed no visible pathomorphological granulomatous changes, the mycobacteria were found through direct microscopy and bacteriological exami-nation. Severe systemic mycobacteriosis without typical granuloma formation was diagnosed by Yanong *et al*. (32) in a group of six mature captive striated frogfish (*Antennarius striatus*), and by Novotny *et al*. (21) in three-spot gourami (*Trichogaster trichopterus*). The presence and distribution of mycobacterial species in clinically healthy aquarium fish and their environ-ment has been also noted by Beran *et al*. (5) and Zanoni *et al*. (33). Moreover, the acid-fast rods were described by Lescenko *et al*. (15) in three out of 26 fish without granulomas. The lack of visible granulomas in the parenchymatous organs does not exclude later pathological processes creating visible granulomas in the organs (15).

The GenoType assay, targeting the 23S rRNA gene region, provides simultaneous identification of 14 different mycobacterial species: *M. avium* subspecies, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*, *M. marinum, M. ulcerans*, *M. peregrinum*, *M. tuberculosis* complex, and *M. xenopi*. Each CM strip contains 17 probes, including amplification and hybridisation controls, to verify the test procedures. Using this test, the isolation of *M. peregrinum* from apparently healthy aquarium fish was possible (Fig. 1). *M. peregrinum* belongs to the *M. fortuitum* complex (the opportunistic pathogens of people and some animal species belonging to the group of rapidly growing, non-tuberculosis mycobacteria), which is widely distributed in the environment and has been reported to be infectious to humans (19, 26). *M. fortuitum*, although resistant to common antituberculosis agents, is often susceptible *in vitro* to antimicrobials, including the quinolones, macrolides, doxycycline, minocycline, and sulfonamides (28). *M. peregrinum* is known to be sensitive to amikacin, ciprofloxacin, faropenem, imipenem, and clarithromycin (26, 30). Differing susceptibility of the

isolates to the antibiotics used in the study suggests the occurrence of different bacterial strains. Further studies must be undertaken to clarify this observation.

In mammals, fast-growing mycobacteria, such as *M. chelonae*, *M. abscessus*, *M. peregrinum,* and *M. fortuitum*, are usually considered opportunistic bacteria and often cause infection in immunocompromised humans (23, 25). Recently, the number of reported cases of infection caused by non-tuberculous mycobacterium has increased, and the clinical importance of these organisms is growing (8, 19, 31). The identification of *M. peregrinum* in fish is significant because it can be a human pathogen that is responsible for infections commonly associated with human skin and soft tissues (19), bacteriaemia (14, 27), pneumonia (16), and tonsillar abscessus (26). This study illustrates that the infection can cause mycobacteriosis in humans. *M. peregrinum* is a significant environ-mental pathogen capable of causing a broad spectrum of diseases. Moreover, the occupational risk for immunodeficient aquaculture operators cannot be ignored.

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