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Occurrence of *Mycobacterium* spp. in ornamental fish

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Abstract

Introduction and objective. Fish mycobacteriosis is a chronic granulomatous disease caused by several species of bacteria from the genus *Mycobacterium,* described as nontuberculous mycobacteria (NTM). The most important species causing fish mycobacterioses are *M. chelonae*, *M. fortuitum*, and *M. marinum*. Mycobacteria infecting fish also include zoonotic pathogens. *M. marinum* is the cause of most cases of fish-related mycobacterial infection in humans. The disease occurs more frequently in workers in the fishing industry, people whose hobbies involve water activities, and aquarists. The aim of the present study was to examine the occurrence of different species of mycobacteria in freshwater ornamental fish.

Materials and method. The occurrence of *Mycobacterium* spp. in freshwater ornamental fish was studied from January 2015 – December 2016. Material isolated from skin scrapings, contents of the digestive tracts, and internal organs of ornamental fish was stained with Ziehl-Neelsen (ZN) and inoculated on Lowenstein-Jensen medium. All isolates found positive by ZN were identified by amplification of the gene encoding the Hsp65 protein. A total of 408 samples obtained from 136 ornamental fish from 36 species were tested.

Results. Using the culture method *Mycobacterium* was isolated from 69 fish (50.1%) and 99 samples (24.3%). Sequence analysis of gene fragments coding for the Hsp65 protein of 99 isolates revealed occurrence of 13 species of mycobacteria: *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. marinum*, *M. mucogenicum*, *M. neoaurum*, *M. peregrinum*, *M. salmoniphilum*, *M. saopaulense*, *M. senegalense*, *M. septicum*, and *M. szulgai*.

Conclusions. The obtained results indicate a significant role of ornamental fish as a source of mycobacteria which are potentially dangerous,especially to humans.

Key words

Mycobacterium spp., ornamental fish, prevalence, public health

INTRODUCTION

Fish mycobacteriosis is caused by several species of bacteria from the genus *Mycobacterium*. Mycobacterial infections are found in both freshwater and marine fish worldwide [1]. The most important species causing fish mycobacterioses are *M. chelonae*, *M. fortuitum*, and *M. marinum* [2]. Other species isolated from fish include *M. abscessus*, *M. arupense*, *M. avium*, *M. chesapeaki*, *M. conceptionense*, *M. flavescens*, *M. gordonae*, *M. haemophilum*, *M. kansasii*, *M. monteforense*, *M. neoaurum*, *M. nonchromogenicum*, *M. parascrofulaceum*, *M. peregrinum*, *M. pseudoshottsii*, *M. salmoniphilum*, *M. saopaulense*, *M. scrofulaceum*, *M. senegalense*, *M. septicum*, *M. shottsii*, *M. simiae*, *M. terrae*, *M. szulgai*, *M. triviale*, *M. triplex*, and *M. xenopi* [3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13].

Fish mycobacteriosis is a chronic disease, often associated with non-specific clinical symptoms. Diseased fish exhibit lethargy, loss of appetite, emaciation, ascites, scale loss, abnormal behaviour, pigment changes, exophthalmia, dermal ulceration, and spinal defects. At necropsy, characteristic grey or white nodules in the muscles and internal organs can be observed [1].

Treatment of fish mycobacteriosis is difficult, cost-consuming, long-lasting, and dangerous to subjects in contact with diseased fish. For this reason, treatment is carried out in cultures of high value. Liquidation is usually recommended of the affected stock and disinfection of the tanks [14].

Mycobacteria infecting fish also include zoonotic pathogens that can cause both localized and disseminated infections in man. The population at risk includes people who are exposed to aquatic environments, mainly workers in the fishing industry and aquarists [1, 15, 16, 17]. The infection usually occurs by contact through wounds caused by infected fish or during the handling of the aquariums, such as cleaning or changing the water [15]. Currently, *M. marinum* causes the most cases of fish related infections in man [15]. However, aquarists should be aware of the zoonotic potential of any of the NTM [16, 17].

Acid fast bacilli (AFB) smear microscopy, despite its lack of specificity, is the first step in the diagnosis of mycobacterial infections [15]. However, due to the difference in clinical significance of NTM, species identification of mycobacterial isolates is necessary. Traditional methods, such as biochemical tests, fail to provide a precise identification of closely related NTM species [18]. Furthermore, molecular methods, such as hybridization DNA probe assays, 16S rRNA gene multiplex PCR, or PCR restriction fragment length polymorphism analysis (PRA) for identification of nontuberculous mycobacteria, might fail to distinguish closely related species [19]. For this reason, gene sequencing is considered the gold standard for identification of *Mycobacterium* species [20]. Gene encoding the 65-kDa, a heat shock protein (*hsp65*) is present in all mycobacterial species and used widely for identification of NTM to species level because of its

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interspecies variability, compared to some other conserved genes such as 16S rRNA or *rpoB* [20, 21].

The zoonotic potential and the great importance of mycobacteriosis for fish breeders highlight the need to characterize the diversity of mycobacteria in ornamental fish.

OBJECTIVE

The aim of the presented study was to examine the occurrence of different species of mycobacteria in diseased freshwater ornamental fish in Poland.

MATERIALS AND METHOD

From January 2015 – December 2016, 136 diseased ornamental fish sent from private aquaria and pet shops to the laboratory of the Department of Fish Diseases and Biology, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Poland, were examined for mycobacteria. Fish were euthanized using an overdose of ethyl 3-aminobenzoate methanesulfonate (Sigma Aldrich, USA). Skin, gut content, and visceral organs (spleen, liver, and kidney) were taken from each fish. If sampling the gut content was not possible due to their small size, entire guts were subjected to the cultivation protocol. Before decontamination, smears of homogenized biological material were prepared, stained with Ziehl-Neelsen (ZN) and observed under 100x (oil immersion) objective lens for the detection of AFB. A negative report was not given untill at least 100 fields had been examined. For mycobacterial culture, the samples were mixed with an equal volume of a 5% oxalic acid solution and incubated for 15 min. Afterwards, the samples were centrifuged at 3,000 g for 15 min. The pellets were washed twice in sterile phosphate buffered saline and inoculated onto one egg Lowenstein-Jensen (LJ) media at 25 °C and 37 °C. The slants were checked daily for 2 months. Clearly visible colonies were examined according to their morphology and confirmed by microscopic examination after ZN staining of smears prepared from the colonies. AFB positive colonies were identified by the amplification of a 439 bp mycobacterial DNA fragment of the *hsp65* gene using primers Tb11 (5′-ACCAACGATGGTGTGTCCAT-3′) and Tb12 (5′-CTTGTCGAACCGCATACCCT-3′) [21]. PCR reactions were performed in a thermal cycler (MJ-Mini, Bio-Rad, USA) with the amplification profile: initial denaturation at 94 °C for 10 min, followed by 45 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were analysed by 1 % agarose gel electrophoresis. DNA fragments of expected length were purified using Gel-Out Extraction Kit (A&A Biotechnology Gdynia, Poland), according to the manufacturer's instructions, and subjected to direct sequencing at a DNA sequencing core facility (Genomed S.A., Warsaw, Poland). DNA sequences were aligned with the available *hsp65* gene sequences from the National Centre for Biotechnology Information Gene Bank for *Mycobacterium* spp. using the MEGA6 software.

RESULTS

During the study, a total of 136 fish were excamined. AFB ZN-stained specimens provided positive microscopic results in 15.44% (n = 21) of the examined fish. Culture methods provided positive results in 69 examined fish (50.74%) and in 99 of the total 408 examined samples (24.26%). All isolates were identified using sequencing of the *hsp65* gene. The identity of mycobacterial isolates according to fish species are shown in Table 1. *Carassius auratus*, *Danio rerio*, *Poecilia reticulate*, *Xiphophorus maculates, Paracheirodon innesi*, *Pterophyllum scalare*, and *Poecilia sphenops* were the most commonly sampled fish. *M. marinum, M. gordonae*, *M. peregrinum*, and *M. chelonae* were the most frequently identified mycobacteria in the above-mentioned fish. *M. marinum* was detected in all of the most commonly sampled fish species with the exception of *C. auratus* and *X. maculates*. Granulomatous lesions suggestive of mycobacteriosis were observed in 5.15% (n = 7) of the fish. Mycobacteria were most frequently isolated from the skin scrapings and contents of digestive tracts, but less frequently from the internal organs (data not shown). A total of 99 mycobacterial isolates were obtained (Tab. 1). The majority of isolates were represented by *M. marinum* (33.33%), *M. chelonae* (16.16%), *M. gordonae* (15.15%), *M. peregrinum* (12.12%), and *M. fortuitum* (10.10%). Other isolates were identified as *M. senegalense* (4.04%), *M. septicum* (2.02%), *M. neoaurum* (2.02%), *M. abscessus* (1.01%), *M. mucogenicum* (1.01%), *M. salmoniphilum* (1.01%), *M. saopaulense* (1.01%), and *M. szulgai* (1.01%). All the sequences obtained from the *hsp65* gene were submitted to the GenBank database under the accession numbers listed in Table 2.

DISCUSSION

Numerous studies have shown that aquaculture organisms are a source of human bacterial infections [15, 16, 17, 22]. Of the bacterial pathogens present in fish and causing human infection, bacteria from the *Mycobacterium* genus are of great importance. A previous study revealed the occurrence mycobacterial infections in 4 species of ornamental fish [23]. Mycobacterial infections in freshwater African catfish (*Clarias gariepinus*) were described by Antychowicz et al. [24]. Besides these reports, little is known about mycobacterial infections in fish in Poland.

In the presented study, AFB were detected by microscopy in 15.44% of the fish and mycobacteria were isolated from 50.74%. Similar results were obtain in Italy by Zanoni et al. [12], who detected AFB in 21.70% of fish and isolated mycobacteria from 35.9% of fish. In the Czech Republic, Lescenko et al. [5] detected AFB in 45.70% of fish and isolated mycobacteria from 70.60% of fish. In another study carried out in the Czech Republic by Beran et al. [3], AFB were detected in 14.30% of fish and mycobacteria were isolated from 42.90% of fish. These researchers also isolated mycobacteria from 75.40% of environmental samples taken from aquariums. In Sweden, Hongslo et al. [25] detected AFB in 23% of fish and isolated mycobacteria from 89% of fish. In Slovenia, Pate et al. [9] detected AFB by microscopy in 37.10% of fish and isolated mycobacteria from 82.90% of fish. In India, Shukla et al. [18] isolated mycobacteria from 25% of examined aquarium fish. One cause of these differences in the NTM smear positivity rates and bacteriology results is the different numbers of mycobacteria in the samples. Other studies support the concept that specimens with low colony counts of mycobacteria are less likely to be detected by smear microscopy [26]. The discrepancy in positive microscopy

Table 1. Overview of ornamental fish investigated and mycobacteria isolated in this study.

Fish	Investigated fish	Positive fish (isolates)	Isolated *Mycobacterium*													No. isolates
			M. abscessus	*M. chelonae*	*M. fortuitum*	*M. gordonae*	*M. marinum*	*M. mucogenicum*	*M. neoaurum*	*M. peregrinum*	*M. salmoniphilum*	*M. saopaulense*	*M. senegalense*	*M. septicum*	*M. szulgai*	
Andinoacara pulcher	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Ameiurus nebulosus	1	1	-	1	-	-	-	-	-	-	1	-	-	-	-	2
Ancistrus multispinis	4	3	-	-	-	-	3	-	-	1	-	-	-	-	-	4
Betta splendens	2	1	-	-	-	-	1	-	-	-	-	-	-	-	-	1
Carassius auratus	12	6	-	3	1	2	-	-	-	-	-	-	-	-	-	6
Chromobotia macracanthus	4	1	-	1	-	-	-	-	-	-	-	-	-	-	-	1
Corydoras punctatus	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Danio rerio	13	8	-	1	1	2	4	-	-	2	-	-	2	-	-	12
Epalzeorhynchos bicolor	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Gymnocorymbus ternetzi	1	1	-	1	-	-	-	-	-	-	-	-	-	-	-	1
Hemigrammus bleheri	2	1	-	-	1	-	-	-	-	-	-	-	-	-	-	1
Labidochromis caeruleus	2	2	-	4	-	-	-	-	-	1	-	-	-	-	-	5
Macropodus opercularis	3	3	-	-	1	-	3	-	-	-	-	-	-	-	-	4
Macrotocinclus affinis	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Mastacembelus erythrotaenia	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Melanochromis cyaneorhabdos	2	1	-	1	-	-	-	-	-	-	-	-	-	-	-	1
Mikrogeophagus ramirezi	3	3	1	1	-	1	1	-	-	1	-	-	-	-	-	5
Maylandia zebra	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Nematobrycon palmeri	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Neolamprologus brichardi	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Pangasius sutchi	1	1	-	-	-	1	-	-	-	-	-	-	-	-	-	1
Paracheirodon innesi	9	4	-	1	1	2	1	-	-	-	-	-	-	-	-	5
Pangio kuhlii	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Pethia nigrofasciata	7	4	-	-	-	2	3	-	-	-	-	-	-	-	-	5
Pethia ticto	1	1	-	-	1	-	-	-	-	-	-	-	-	-	-	1
Placidochromis platyrhynchos	1	1	-	1	-	-	-	-	-	1	-	-	1	-	-	3
Poecilia reticulata	12	7	-	-	1	3	2	-	2	1	-	-	-	-	-	9
Poecilia sphenops	8	3	-	-	-	-	1	1	-	-	-	-	-	-	1	3
Pterophyllum scalare	8	4	-	1	-	-	3	-	-	2	-	-	1	-	-	7
Symphysodon discus	3	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Tanichthys albonubes	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Trichogaster lalius	3	2	-	-	-	-	5	-	-	-	-	-	-	-	-	5
Trichopodus trichopterus	4	4	-	-	2	1	1	-	-	1	-	1	-	-	-	6
Trigonostigma heteromorpha	2	1	-	-	-	-	-	-	-	-	-	-	-	1	-	1
Xiphophorus maculatus	12	4	-	-	1	1	-	-	-	2	-	-	-	1	-	5
Xiphophorus hellerii	6	2	-	-	-	-	5	-	-	-	-	-	-	-	-	5
Total	136	69	1	16	10	15	33	1	2	12	1	1	4	2	1	99

results and bacteriology results could also relate in part to the differences in acid-alcohol-fastness, which tends to be a stronger attribute in slow-growing mycobacteria [27].

All 13 species of mycobacteria isolated in the present study were previously isolated from ornamental fish. In the Czech Republic, studies on the occurrence of mycobacteria in aquarium fish were conducted by Lescenko et al. [5] in which the isolated mycobacteria were represented by the species *M. marinum*, *M. gordonae*, *M. triviale*, and *M. avium* subsp. *hominissuis*. Similar studies, also in the Czech Republic, were conducted by Beran et al. [3], who isolated *M. fortuitum*, *M. flavescens*, *M. chelonae*, *M. gordonae*, *M. terrae*, *M. triviale*, *M. diernhoferi*, *M. celatum*, *M. kansasii*, and *M. intracellulare*. The most frequently isolated mycobacteria were *M. fortuitum*,

Table 2. *Mycobacterium* strains identified by sequencing of *hsp65* PCR products. *M. abscessus* (*M. a.*), *M. chelonae* (*M. c.*), *M. fortuitum* (*M. f.*), *M. gordonae* (*M. g.*), *M. marinum* (*M. m.*), *M. mucogenicum* (*M. muc.*), *M. neoaurum* (*M. n.*), *M. peregrinum* (*M. p.*), *M. salmoniphilum* (*M. sal.*), *M. saopaulense* (*M. sao.*), *M. senegalense* (*M. sen.*), *M. septicum* (*M. sep.*), *M. szulgai* (*M. sz.*)

Species	Strain	Accession No.	Source	Species	Strain	Accession No.	Source
M. a.	M11	KX231724	*M. ramirezi*	*M. m*	M21	KX231690	*T. lalius*
M. c.	M2	KX231726	*L. caeruleus*	*M. m*	M26	KX231691	*A. multispinis*
M. c.	M8	KX231727	*P. scalare*	*M. m*	M46	KX231692	*A. multispinis*
M. c.	M23	KX231729	*C. auratus*	*M. m*	M47	KX231693	*A. multispinis*
M. c.	M34	KX231730	*C. auratus*	*M. m*	M48	KX231694	*P. reticulata*
M. c.	M36	KX231731	*C. auratus*	*M. m*	M50	KX231695	*P. sphenops*
M. c.	M37	KX231732	*A. nebulosus*	*M. m*	M55	KX231698	*D. rerio*
M. c.	M40	KX231733	*M. cyaneorhabdos*	*M. m*	M56	KX231699	*D. rerio*
M. c.	M83	KX231735	*D. rerio*	*M. m*	M58	KX231700	*M. ramirezi*
M. c.	M94	KX231736	*G. ternetzi*	*M. m*	M60	KX231702	*M. opercularis*
M. c.	M97	KX231737	*P. platyrhynchos*	*M. m*	M62	KX231703	*B. splendens*
M. c.	M100	KX231738	*L. caeruleus*	*M. m*	M70	KX231706	*M. opercularis*
M. c.	M101	KX231739	*L. caeruleus*	*M. m*	M72	KX231707	*D. rerio*
M. c.	M102	KX231740	*L. caeruleus*	*M. m*	M90	KX231708	*T. trichopterus*
M. c.	M12	KX244857	*C. macracanthus*	*M. m*	M53	KX231696	*P. nigrofasciata*
M. c.	M18	KX231728	*P. innesi*	*M. m*	M54	KX231697	*P. innesi*
M. c.	M57	KX231734	*M. ramirezi*	*M. m*	M59	KX231701	*D. rerio*
M. f.	M7	KX244856	*P. reticulata*	*M. m*	M67	KX231704	*P. nigrofasciata*
M. f.	M14W	KX231765	*M. opercularis*	*M. m*	M68	KX231705	*P. nigrofasciata*
M. f.	M17	KX231766	*P. innesi*	*M. m*	M42	KX231751	*X. maculatus*
M. f.	M22	KX244858	*H. bleheri*	*M. m*	M43	KX231752	*X. maculatus*
M. f.	M29	KX231767	*C. auratus*	*M. m*	M49	KX231753	*P. reticulata*
M. f.	M52	KX231768	*X. maculatus*	*M. m*	M63	KX231754	*P. scalare*
M. f.	M81	KX231769	*D. rerio*	*M. m*	M64	KX231755	*P. scalare*
M. f.	M88	KX231770	*T. trichopterus*	*M. m*	M84	KX231756	*P. scalare*
M. f.	M91	KX231771	*P. ticto*	*M. muc.*	M6	KX244855	*P. nigrofasciata*
M. f.	M92	KX231772	*T. trichopterus*	*M. n.*	M75	KX244862	*P. reticulata*
M. g.	M25	KX231711	*C. auratus*	*M. n.*	M76	KX244863	*P. reticulata*
M. g.	M27	KX231712	*P. sutchi*	*M. p.*	M24	KX231742	*M. ramirezi*
M. g.	M28	KX231713	*P. reticulata*	*M. p.*	M45	KX231743	*D. rerio*
M. g.	M30	KX231714	*P. innesi*	*M. p.*	M71	KX231744	*A. multispinis*
M. g.	M61	KX244861	*C. auratus*	*M. p.*	M79	KX231745	*D. rerio*
M. g.	M66	KX231719	*X. maculatus*	*M. p.*	M82	KX231746	*X. maculatus*
M. g.	M19	KX231709	*D. rerio*	*M. p.*	M98	KX231749	*P. scalare*
M. g.	M20	KX231710	*D. rerio*	*M. p.*	M99	KX231750	*P. scalare*
M. g.	M31	KX231715	*T. trichopterus*	*M. p.*	M73	KX231758	*P. reticulata*
M. g.	M32	KX231716	*M. ramirezi*	*M. p.*	M78	KX231759	*X. maculatus*
M. g.	M33	KX231717	*C. auratus*	*M. p.*	M96	KX231748	*P. platyrhynchos*
M. g.	M65	KX231718	*P. nigrofasciata*	*M. p.*	M1	KX231741	*L. caeruleus*
M. g.	M85	KX231720	*P. reticulata*	*M. p.*	M93	KX231747	*T. trichopterus*
M. g.	M86	KX231721	*P. reticulata*	*M. sal.*	M39	KX244859	*A. nebulosus*
M. g.	M87	KX231722	*P. nigrofasciata*	*M. sao.*	M89	KX231725	*T. trichopterus*
M. m	M3	KX231683	*T. lalius*	*M. sen.*	M74	KX231761	*P. scalare*
M. m	M4	KX231684	*T. lalius*	*M. sen.*	M95	KX231762	*P. platyrhynchos*
M. m	M9	KX231685	*X. hellerii*	*M. sen.*	M80	KX231763	*D. rerio*
M. m	M10	KX231686	*X. hellerii*	*M. sen.*	M41	KX231764	*D. rerio*
M. m	M13	KX231687	*X. hellerii*	*M. sep.*	M51	KX244860	*T. heteromorpha*
M. m	M14	KX231757	*M. opercularis*	*M. sep.*	M77	KX231760	*X. maculatus*
M. m	M15	KX231688	*T. lalius*	*M. sz.*	M69	KX231723	*P. sphenops*
M. m	M16	KX231689	*T. lalius*				

M. flavescens, *M. chelonae*. However, they did not isolate *M. marinum*, i.e. a species that was most frequently identified in the presented study. However, in studies carried out in Sweden by Hongslo et al. [25], the most frequently isolated species was *Mycobacterium marinum*, followed by *M. chelonae* and *M. gordonae*. In Italy, Zanoni et al. [12] isolated *M. fortuitum*, *M. peregrinum*, *M. chelonae*, *M. abscessus*, *M. marinum*, *M. gordonae*, *M. nonchromogenicum*, and *M. interjectum*. The most frequently isolated mycobacteria were *M. fortuitum*, *M. peregrinum*, and *M. chelonae*. In India, Shukla et al. [18] isolated *M. abscessus*, *M. gordonae*, *M. fortuitum*, *M. conceptionense*, *M. parascrofulaceum*, and *M. senegalense*. The most frequently isolated mycobacteria were *M. abscessus* and *M. gordonae*.

The difference in the prevalence of mycobacteria among different countries could relate to the endemic occurrence of certain species of mycobacteria in water-supply systems. For example, the Czech Republic is a country with an endemic incidence of *M. kansasii* in water [3]. The variation in prevalence may also depend on the fish supplier. Smith et al. [28] suggest that cleaning regimes, filtration, or handling procedures influence the diversity of bacteria within the tanks. The differences in the mycobacterial isolation rate may also depend on the diversity of fish species from which samples were taken. Numerous species of ornamental fish have been reported with mycobacterial infections; however, some species appear to be more susceptible and therefore demonstrate a higher incidence of infections [12].

The present study has shown that aquarium fish are a source of mycobacteria, which are potentially pathogenic for both fish and humans. It is noteworthy that *M. marinum*, i.e. the most frequently isolated species in this work, causes most cases of fish-related mycobacterial infection in humans.

Cconflict of interest

The authors declare that they have no competing interests.

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