Occurrence of different species of mycobacteria in aquarium fish from Swedish pet-shops

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Abstract

In a health survey of freshwater aquarium fish from 24 Swedish pet-shops, carried out 2006 to 2007, one of the most common causes of diseases identified was infection with acid-fast bacteria. By microscopic investigation 28 (23%) out of 120 aquarium fish with signs of illness were found to have probable or established infection with acid-fast bacteria. The aim of the present study was to examine the occurrence of mycobacteria in the aquarium fish samples from the 24 Swedish pet-shops previously studied and also to characterize the acid-fast bacteria found in the 28 aquarium fish with signs of illness to the species level. A pool consisting of organ materials from 10 fish from each of the 24 pet-shops and tissue samples from each of the 28 individual fish were investigated, using direct sequencing of the 16S rRNA gene or cultivation in combination with 16S rRNA gene sequencing. The most common mycobacteria species found in the pooled samples from the pet-shops was *Mycobacterium marinum*, which was detected in 50% of the samples, followed by *M. fortuitum* and *M. peregrinum*, which both were detected in 29% of the samples. The investigation of individual fish demonstrated that at least one mycobacteria species was present in 25 (89%) out of the 28 fish and that the most common mycobacteria species also detected here was *M. marinum* that occurred in 15 (54%) of the fish. Other mycobacteria species identified in the individual fish were *M. arupense, M. chelonae, M. florentinum, M. fortuitum, M. gordonae* and *M. haemophilum*. In summary, 8 species of mycobacteria were identified in freshwater aquarium fish from Swedish pet-shops, species that all have been described to cause disease in fish, but also to be zoonotic.

Introduction

Keeping fish as pets in aquaria is a popular hobby in Sweden. However, aquarium fish can get problems with infections, there especially bacteria belonging to the genus *Mycobacterium* have been reported to cause serious disease (Puttinaowarat et al., 2000; Sakai et al., 2005). Several studies have been published regarding occurrence of mycobacteria or acid-fast bacteria in aquarium fish sampled from fish breeders, wholesalers and private aquarists (Beran et al., 2006; Lescenko et al., 2003; Novotny et al., 2010; Prearo et al., 2004; Zanoni et al., 2008). However, only a few previous studies have examined the occurrence of mycobacteria or acid-fast bacteria in aquarium fish from pet-shops (Gomez, 2008; Pate et al., 2005; Wickins et al., 2011). A previous health survey of freshwater aquarium fish from 24 Swedish pet-shops, carried out 2006 to 2007, indicated that one of the most common causes of diseases was infection with acid-fast bacteria (Hongslo & Jansson, 2009). By thorough microscopic investigation 28 (23%) of 120 aquarium fish with signs of illness were found to have probable or established infection

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with acid-fast bacteria. The aim of the present study was to examine the occurrence of mycobacteria in the aquarium fish samples from the 24 Swedish pet-shops previously studied and also to characterize the acid-fast bacteria found in the 28 individual fish with signs of illness to the species level, using cultivation of bacteria and/ or molecular genetic techniques.

Materials and methods

To determine the occurrence of mycobacteria in the samples previously collected from the 24 Swedish pet-shops, one pooled sample consisting of organ materials from 10 freshwater fish (i.e. one fish from 10 different aquariums) from each of the pet-shops (Hongslo & Jansson, 2009) was investigated. Fish had primarily been selected according to signs of illness, otherwise fish had been randomly collected. The 24 pooled samples had been homogenized in Earle's Minimal Essential Medium supplemented with 10% calf serum without antibiotics and the received sediments had been kept in -70°C until start of this study. To avoid growth of bacteria other than acid-fast bacteria, the sediments were treated with N-acetyl-L-cysteine-sodium hydroxide before cultivation (Clinical and Laboratory Standards Institute, 2008). Cultivation of the 24 sediments were then carried out on the following media: Löwenstein-Jensen supplemented with glycerol (7.5 mL/ L), Löwenstein-Jensen supplemented with alanine (1.5 g/ L) and galactose (40 g/ L), Middlebrook 7H10 with albumin fraction V, dextrose and catalase enrichment and Stonebrink (Clinical and Laboratory Standards Institute, 2008). The cultivation tubes were incubated at 20°C, 30°C and 37°C (in total 24x4x3 tubes) for two months and examined weekly for detection of possible acid-fast bacteria. In each of the 24 cultivated sediments, one to two acid-fast bacteria colonies were chosen according to colony morphology and pigmentation. These acid-fast bacteria colonies were sub-cultured and frozen in -20°C until analysis. The acid-fast bacteria were then homogenized in 500 µL TE buffer (pH 8), using a Tissuelyser (Qiagen, Hilden, Germany) and 200 µL 0.1 mm Zirconia/ Silica beads (Bio Spec Products Inc., Bartlesville, OK, USA), followed by DNA extraction with the Biorobot EZ1 robot (Qiagen, Hilden, Germany). Thereafter, the 16S rRNA gene was amplified from prepared DNA with selected primers and sequenced as previously described (Johansson et al., 2006). The consensus sequence was matched using the databases GenBank (http://www.ncbi.nlm.nih.gov/) and RDP (http://rdp.cme.msu.edu/) and the softwares BLASTn and SeqMatch, respectively.

To determine acid-fast bacteria of the earlier collected and frozen (-20°C) 28 freshwater aquarium fish to the species level, granulomatous tissue from the kidney, liver, mesenterium or spleen, or skin wounds from each fish was homogenized in 180 µL ATL buffer (Qiagen, Hilden, Germany), using a BeadBeater (MP Biomedicals Fastprep24) and 100 µL 0.1 mm Zirconia/ Silica beads (Bio Spec Products Inc., Bartlesville, OK, USA). DNA was extracted from homogenized fish tissue, using QIAamp DNA Mini kit according to Qiagen's recommendations. In fish there no bacterial DNA was detected in homogenized tissue samples (n=10), tissue samples were also cultivated on the media described above (Clinical and Laboratory Standards Institute, 2008) and on an additional 7H10 Middlebrook media with oleic acid, albumin, dextrose and catalase enrichment and 60 µM hemin supplement (Dawson and Jennis,

1980; Vadney and Hawkins, 1985; Whipps et al., 2007), followed by DNA extraction from selected bacterial colonies.

The DNA samples were amplified using primers for the 16S rRNA gene described in Table 1. The PCR reaction was performed in a total volume of 10 µL in a 96-Multiply PCR plate nature (Sarstedt, Numbrecht, Germany) on a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, USA). Sequencing of the 16S rRNA gene was made with BigDyeTerminator v3.1 (Applied Biosystems, Foster City, USA). Analysis of data was performed with the software 3730 Data Collection v3. Consensus sequences were obtained by alignment of forward and reverse reads using CodonCode Aligner 3.631. Editing and alignment of sequences were performed with gi|57116681:1471846-1473382 *M. tuberculosis* as reference sequence. The consensus sequence was matched using the database RDP (http://rdp.cme.msu.edu/) and the software SeqMatch.

Results

Five species of mycobacteria were identified in aquarium fish from the 24 Swedish pet-shops (Table 2). One mycobacteria species was detected in each of 14 (58%) of the 24 pet-shops and two were detected in 10 (42%) of the pet-shops. The most frequent mycobacteria species found in the pet-shops was *M. marinum*, which was detected in 50% of the pet-shops, followed by *M. fortuitum* and *M. peregrinum*, which both were detected in 29% of the pet-shops. Less frequent species were *M. gordonae* and *M. chelonae*, which were detected in 21% and 13% of the pet-shops, respectively. When comparing the occurrence of mycobacteria species in pet-shops selling both aquarium fish and other animals (n=12) with that in pet-shops selling only aquarium fish (n=12) (Table 2), no significantly differences were found, either in number or type of mycobacteria species in the pet-shops (data not shown).

The mycobacteria species found in the 28 aquarium fish with probable or established infection with acid-fast bacteria are given in Table 3. The investigation demonstrated presence of one mycobacteria species in each of 22 (79%) of the 28 fish and a combination of mycobacteria species in 3 (11%) of the fish. In 3 (11%) of the 28 fish, no mycobacteria species could be detected. Four of the 28 fish with probable or established infection with acid-fast bacteria were not included in any of the pooled samples (Table 3:

Table 1. Primers used for PCR and sequencing of the 16S rRNA gene of *Mycobacteria* spp.

Designation (direction)	Position[a]	Sequence	Application
Forward	4 - 25	GCGGCGTGCTTAACACATGCAA	PCR/Sequencing
Reverse	1476 - 1452	TTCCGGTACGGCTACCTTGTTACGA	PCR/Sequencing
Forward	503 - 526	TGTCCGGAATTACTGGGCGTAAAG	Sequencing
Forward	1131 - 1153	AAGGTGGGGATGACGTCAAGTCA	Sequencing
Reverse	523 - 500	TACGCCCAGTAATTCCGGACAACG	Sequencing
Reverse	990 - 968	AGGCCACAAGGGAACGCCTATCT	Sequencing

[a]Primer design and positions according to the *Mycobacterium ulcerans* 16S rRNA gene sequence

Table 2. Identified mycobacteria species in pooled samples from aquarium fish in 24 Swedish pet-shops.

Pet-shop[a]	Mycobacteria species	Sequenced bases (n)[b]
1	*M. chelonae*	1420
	M. marinum	1435
2	*M. fortuitum*	1422
	M. marinum	1435
3	*M. chelonae*	1420
4	*M. gordonae*	1434
	M. peregrinum	1422
5	*M. peregrinum*	1422
6	*M. fortuitum*	1422
	M. gordonae	1434
7	*M. fortuitum*	1343[c]
	M. peregrinum	1422
8	*M. fortuitum*	1422
9	*M. marinum*	1435
	M. peregrinum	1422
10	*M. marinum*	1435
11	*M. gordonae*	1434
12	*M. gordonae*	1356[c]
	M. marinum	1319[c]
13	*M. peregrinum*	1332[c]
14	*M. marinum*	1435
15	*M. chelonae*	1420
	M. marinum	1435
16	*M. gordonae*	1434
17	*M. marinum*	1435
18	*M. marinum*	1435
19	*M. peregrinum*	1422
20	*M. fortuitum*	1422
21	*M. marinum*	1435
	M. peregrinum	1422
22	*M. fortuitum*	1422
	M. marinum	1435
23	*M. marinum*	1435
24	*M. fortuitum*	1422

[a]1-12 = pet-shops selling both aquarium fish and other animals; 13-24 = pet-shops selling only aquarium fish.
[b]Matched using the databases GenBank (http://www.ncbi.nlm.nih.gov/) and RDP release 10 (http://rdp.cme.msu.edu/) and the softwares BLASTn and SeqMatch.
[c]Matched using only the database RDP release 10 (http://rdp.cme.msu.edu/) and the software SeqMatch.

fish no 5, 6, 15 and 22). However, out of the other 24 fish, 13 (54%) showed mycobacteria species that were in accordance with the mycobacteria species found in the pooled samples (Table 3).

Discussion

This study demonstrated that the most common species of mycobacteria in Swedish pet-shops in 2006-2007 was *M. marinum*, which was detected in 50% of the pooled samples from the 24 pet-shops investigated, followed by *M. fortuitum* and *M. peregrinum* which were both found in 29% of the pooled samples. In the 28 aquarium fish collected from 18 of the pet-shops, the most common mycobacteria species was also *M. marinum* occurring in 15 (54%) of the fish, followed by *M. peregrinum* in 3 (11%) and *M. fortuitum* in 2 (7%) of the fish. These findings are important as all three mycobacteria species are known to cause disease in fish (Harriff et al., 2007; Puttinaowarat et al., 2000) and are known to cause infections in man (Jernigan & Farr, 2000; Kamijo et al., 2012; Kothavade et al., 2013). The species *M. chelonae*, *M. florentinum* and *M. gordonae* have also been reported to be pathogenic for both fish as well as humans (Asija et al., 2011; Astrofsky et al., 2000; Kothavade et al., 2013; Pourahmad et al., 2008; Sakai et al., 2005; Tortoli et al., 2005), but occurred less frequently in this study. *M. arupense* was detected in 1 (4%) of the 28 fish tested and this is to the best of our

Table 3. Mycobacteria species identified in the 28 aquarium fish with probable or established infection with acid-fast bacteria.

No[a]	Fish group	Fish species	Mycobacteria species	Sequenced bases (n)	Match[b] (%)
1[c] [23]	Live bearers	Platy *(Xiphophorus maculatus)*	*M. marinum*	1379	99.7
2[e] [3]	Cyprinids	Cherry barb *(Puntius titteya)*	*M. marinum*	1360	100
3 [8]	Cyprinids	Cherry barb *(Puntius titteya)*	*M. marinum*	1383	99.9
4[c] [18]	Cyprinids	Comet goldfish *(Carassius auratus auratus)*	*M. marinum*	1332	99.9
5[e] [–]	Cyprinids	Fantail goldfish *(Carassius auratus auratus)*	*M. gordonae* *M. peregrinum*	1350 1251	100 100
6[e] [–]	Cyprinids	Fantail goldfish *(Carassius auratus auratus)*	*M. fortuitum*	1320	100
7[e] [4]	Cyprinids	Tiger barb *(Puntius tetrazona)*	No mycobacteria found	-	-
8[d,e] [7]	Labyrinth fish	Cosby gourami *(Trichogaster trichopterus)*	*M. arupense* *M. peregrinum*	1334 1336	100 100
9 [8]	Labyrinth fish	Dwarf gourami *(Colisa lalia)*	*M. marinum*	1384	99.9
10[c,e] [10]	Labyrinth fish	Honey gourami *(Colisa chuna)*	*M. marinum*	1351	99.7
11[c] [22]	Labyrinth fish	Siamese fighting fish *(Betta splendens)*	*M. marinum*	1390	99.4
12[c] [23]	Labyrinth fish	Siamese fighting fish *(Betta splendens)*	*M. marinum*	1339	99.9
13[e] [16]	Labyrinth fish	Snakeskin gourami *(Trichogaster pectoralis)*	*M. fortuitum* *M. peregrinum*	1311 1332	100 99.9
14 [13]	Labyrinth fish	Thick-lipped gourami *(Colisa labiosus)*	*M. marinum*	1362	99.3
15[e] [–]	Cichlids	Angelfish *(Pterophyllum scalare)*	No mycobacteria found	-	-
16[e] [11]	Cichlids	Labidochromis yellow *(Labidochromis caeruleus)*	*M. chelonae*	1334	100
17[c] [14]	Cichlids	Rainbow krib *(Pelvicachromis pulcher)*	*M. marinum*	1375	99.9
18[e] [6]	Tetras	Black tetra *(Gymnocorymbus ternetzi)*	No mycobacteria found	-	-
19[c,e] [6]	Tetras	Cardinal tetra *(Paracheirodon axelrodi)*	*M. gordonae*	1324	100
20[c] [1]	Tetras	Colombian tetra *(Hyphessobrycon columbianus)*	*M. marinum*	1384	99.6
21[c] [18]	Tetras	Colombian tetra *(Hyphessobrycon columbianus)*	*M. marinum*	1393	99.2
22 [–]	Tetras	Cuchu's blue tetra *(Boehlkea fredcochui)*	*M. haemophilum*	1374	98.9[f]
23[c] [1]	Tetras	Glowlight tetra *(Hemigrammus erythrozonus)*	*M. chelonae*	1371	99.8
24[c] [17]	Tetras	Glowlight tetra *(Hemigrammus erythrozonus)*	*M. marinum*	1362	99.9
25[c] [21]	Tetras	Glowlight tetra *(Hemigrammus erythrozonus)*	*M. marinum*	1380	99.5
26 [2]	Tetras	Swegles tetra *(Hyphessobrycon sweglesi)*	*M. florentinum*	1366	97.4
27[c] [12]	Rainbow fish	Red rainbow fish *(Glossolepis incisus)*	*M. marinum*	1383	99.9
28 [23]	Other fish	Peacook gudgeon *(Tateurdina ocellicauda)*	*M. haemophilum*	1374	99.0[f]

[a]The pet-shop number is given in square brackets [ ] and is the same as in Table 2. The square brackets without number [–] means a fish not included in the pooled samples.
[b]Matched by the database RDP release 10 (http://rdp.cme.msu.edu/) and the software SeqMatch.
[c]In accordance with mycobacteria species found in the pooled sample.
[d]Partly in accordance with mycobacteria species found in the pooled sample.
[e]Cultivated.
[f]Matched by the database RDP release 11 (http://rdp.cme.msu.edu/) and the software SeqMatch.

knowledge the first report of this mycobacteria species in a diseased aquarium fish. *M. arupense* has, however, recently been isolated from the gills of European tench (*Tinca tinca*) collected from a fish pond (Slany et al., 2014) as well as being reported as a pathogenic agent in man (Legout et al., 2012).

The main difference between the results from the 24 pet-shops and the 28 separate fish sampled was that *M. haemophilum* was detected in two (7%) of the 28 fish by direct 16S rRNA gene sequencing of organ tissues, but this species was not detected in any of the pooled samples from the pet-shops which all were cultivated before 16S rRNA sequencing of grown bacterial colonies. This difference is probably due to the fact that the pooled samples from the pet-shops were not cultivated on fully appropriate media for cultivating *M. haemophilum*, as the samples were cultivated on media without hemin supplement (Dawson and Jennis, 1980; Vadney and Hawkins, 1985). *M. haemophilum* has been reported to cause significant health problem in zebrafish (*Danio rerio*) in research facilities (Whipps et al., 2007). This mycobacteria species has also been recognized as a cause of disease in humans (Bruijnesteijn van Coppenraet et al., 2005; Straus et al., 1994).

Few other studies regarding the occurrence of acid-fast bacteria or mycobacteria identified to the species level, in aquarium fish, in pet-shops have been published (Gomez, 2008; Pate et al., 2005; Wickins et al., 2011). In the study by Gomez (2008) from Spain regarding 200 aquarium fish with clinical signs of chronic disease from pet-shops and private owners, granulomatous inflammation associated with acid-fast bacteria was confirmed histologically in 81 (41%) of the fish, which is doubled that observed in the current. In another study by Wickins and colleagues (2011) regarding 108 aquarium fish with signs of disease from 24 pet-shops in Australia, only 7.4% of the fish showed presence of acid-fast bacteria using microscopy and Ziehl-Neelsen staining. However, the acid-fast bacteria were not determined to the species level in either of these two studies. A possible explanation for the difference in frequency of acid-fast bacteria in the fish between our study and these two others (Gomez 2008; Wickins et al., 2011) is the difference in criteria for selection of the fish investigated. In our study, fish with signs of acute or chronic illness were selected for investigation, whereas in the study by Gomez (2008) only fish with signs of chronic disease were chosen, which could have contributed to the higher frequency of granulomas and acid-fast bacteria compared to in our study. In the study by Wickins et al (2011) wider criteria in the selection of the diseased fish were used which could explain their lower frequency of fish with acid-fast bacteria than in our study. The study by Pate et al (2005), aiming to identify the species of mycobacteria isolated from 35

aquarium fish from pet-shops or private owners in Slovenia, revealed positive microscopy results regarding acid-fast bacteria in 13 (37%) of the fish. These authors used culture in combination with molecular genetic methods and mycobacteria species could be identified in as many as 29 (83%) of the fish. In their study, the most common mycobacteria species identified were *M. fortuitum* in 8 (23%) and *M. marinum* in 7 (20%) of the 35 fish, which can be compared to the results in our study, showing *M. marinum* in 15 (54%) and *M. fortuitum* only in 2 (7%) of the 28 fish.

In summary, different mycobacteria species were identified in pooled samples from all of the 24 pet-shops investigated and in 25 (21%) of the 120 aquarium fish with signs of illness. The most common mycobacteria species found were *M. marinum* (in 50% of the pet-shops and separate fish investigated), followed by *M. peregrinum*, *M. fortuitum, M. gordonae and M. chelonae*. All these mycobacteria species are known to cause disease in fish and to have the ability to infect man.

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