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# Detection of mycobacteria in aquarium fish in Slovenia by culture and molecular methods

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ABSTRACT: Thirty-five aquarium fish were investigated for the presence of mycobacteria by culture and molecular methods. The following species were examined: goldfish *Carassius auratus auratus*, guppy *Poecilia reticulata*, 4 three-spot gourami *Trichogaster trichopterus*, dwarf gourami *Colisa lalia*, Siamese fighting fish *Betta splendens*, freshwater angelfish *Pterophyllum scalare*, African cichlid fish *Cichlidae* spp., cichlid fish *Microgeophagus altispinosus*, cichlid fish *Pseudotropheus lombardoi*, blue streak hap *Labidochromis caeruleus*, sterlet *Acipenser ruthenus*, southern platyfish *Xiphophorus maculatus*, and catfish *Corydoras* spp. Isolates of mycobacteria were obtained in 29 cases (82.9%). Two specimens were positive using Ziehl-Neelsen (ZN) staining, but the cultivation failed. Four specimens were both ZN- and culture-negative. On the basis of GenoType *Mycobacterium* assay (Hain Lifescience) and restriction enzyme analysis of the amplified products (PCR-RFLP), 23 isolates (79.3%) were identified: 7 as *Mycobacterium fortuitum*, 6 as *M. gordonae*, 6 as *M. marinum*, 3 as *M. chelonae*, and 1 as *M. peregrinum*. Five isolates remained unidentified (*Mycobacterium* spp.). One case probably represented a mixed infection (*M. marinum*/*M. fortuitum*). Since *M. marinum* infections are also detected in humans, the significance of mycobacteria in aquarium fish should not be overlooked.

KEY WORDS: Mycobacteria · Aquarium fish · PCR · Restriction enzyme analysis



## INTRODUCTION

Mycobacterial infections are one of the most common infections of aquarium fish. Since their initial discovery at the end of 19th century (Bataillon et al. 1897), fish mycobacterial diseases have been reported to occur worldwide in more than 150 species (Vogel 1958, Nigrelli & Vogel 1963). The mycobacteria that are generally accepted as fish pathogens are *Mycobacterium marinum*, *M. fortuitum* and *M. chelonae*. Several other species are listed in the literature but most isolates belong to one of these species (Austin & Austin 1989).

The disease, usually referred to as fish tuberculosis or mycobacteriosis, has a chronic progressive character. Clinical signs are variable, depending on the main sites of the infection and its severity. Infected fish may appear normal and infection may not be suspected until fish colouration fades and movements become sluggish. Clinical signs also include open lesions and ulcerations, skin inflammation, exophthalmia, and signs of emaciation. Tubercles may occur in all internal organs, but are usually seen on the liver, kidney and spleen (Dulin 1979, van Duijn 1981).

In addition to fish tuberculosis, mycobacteria are capable of causing both localized and disseminated infections in man (Engbaek et al. 1980, Collins et al. 1985, Huminer et al. 1986, Edelstein 1994, Jernigan & Farr 2000, Enzensberger et al. 2002, Lewis et al. 2003). The risk population involves workers in the seafood industry, people whose hobbies involve water activities and fish enthusiasts. *Mycobacterium fortuitum* and *M. chelonae* usually cause superficial lesions via skin wounds, but pulmonary disease and cervical lymph node infection may also occur. Most *M. marinum* infections are cutaneous, with the hands being commonly affected (Lucas 1989). The proportion of aquarium-related *M. marinum* infections in humans reported by Jernigan & Farr (2000) was 49.2%. Due to the zoonotic

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character of the disease, any suspicion of fish mycobacteriosis should be taken seriously and investigation of the case conducted.

The diagnosis of mycobacterial disease in fish is based on histopathological, culture and molecular methods. The aim of the present study was to identify the species of mycobacteria isolated from aquarium fish in Slovenia in the period from 2001 to 2004 and to compare the available identification methods in order to assess their suitability for routine use.

## MATERIALS AND METHODS

**Materials.** The internal organs of 35 aquarium fish were investigated. The number of fish and the species investigated were: 11 goldfish *Carassius auratus auratus*, 7 freshwater angelfish *Pterophyllum scalare*, 4 three-spot gourami *Trichogaster trichopterus*, 3 guppy *Poecilia reticulata*, 2 southern platyfish *Xiphophorus maculatus*, 1 dwarf gourami *Colisa lalia*, 1 Siamese fighting fish *Betta splendens*, 1 African cichlid fish *Cichlidae* spp., 1 cichlid fish *Microgeophagus altispinosus*, 1 cichlid fish *Pseudotropheus lombardoi*, 1 blue streak hap *Labidochromis caeruleus*, 1 sterlet *Acipenser ruthenus*, and 1 catfish *Corydoras* spp.

**Bacteriology.** Smears made directly from fish organs were stained with Ziehl-Neelsen (ZN). Following homogenization, decontamination with N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH), and concentration of the specimens, their sediments were inoculated on the following media: Stonebrink, Middlebrook 7H10, Löwenstein-Jensen with pyruvate, Löwenstein-Jensen with glycerine and Mycobacteria Growth Incubator Tube (MGIT; Becton Dickinson) (Kent & Kubica 1985). The slants were incubated at 37°C, 30°C, and at room temperature for 2 mo. The isolates were subjected to physical and biochemical examination in order to identify the species of mycobacteria. The rate of growth at different temperatures, colony morphology and pigmentation were evaluated. Biochemical methods included growth on MacConkey agar, tolerance to 5% NaCl, and the following enzymatic activities: nitrate reductase, semiquantitative catalase at 37°C, heat-stable catalase at 68°C, Tween 80 hydrolysis, potassium tellurite reductase, arylsulfatase, pyrazinamidase and urease.

**GenoType *Mycobacterium* assay.** GenoType *Mycobacterium* (GTM) molecular genetic assay (Hain Lifescience) was also used for identification of mycobacterial species from cultured material. The test is based on the DNA STRIP® technology and permits the identification of 13 mycobacterial species. The procedure is divided into 3 steps: (1) DNA isolation from cultured material, (2) amplification with biotinylated primers, and (3) reverse hybridization. The hybridization includes chemical denaturation of the amplification product, hybridization of the single-stranded, biotin-labelled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate, and an AP-mediated staining reaction. The assay was performed according to the manufacturer's instructions.

**AccuProbe *Mycobacterium gordonae* assay.** The AccuProbe *Mycobacterium gordonae* culture identification test (Gen-Probe) uses a single-stranded DNA probe with a chemiluminescent label that is complementary to the rRNA of the target organism. The labelled DNA probe and the target organism's rRNA combine to form a stable DNA:RNA hybrid. The selection reagent allows the differentiation between non-hybridized and hybridized probe. The labelled DNA:RNA hybrids are measured in a luminometer.

**Restriction enzyme analysis of PCR products (PCR-RFLP).** DNA was extracted using a simplified isolation procedure as follows: a loop full of bacterial culture was suspended in 50 µl PCR-grade water (Invitrogen), heated for 15 min at 100°C and then centrifuged for 2 min at 14 000 × *g*. One µl of the supernatant was used for PCR.

A set of previously described genus-specific oligonucleotide primers was used to amplify a 924 bp fragment from the 16S rRNA gene of *Mycobacterium* spp. Additional internal primers were used to amplify a 300 bp DNA fragment (Talaat et al. 1997). PCR amplification was performed in a GeneAmp PCR system 2400 (Applied Biosystems). PCR products were analysed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Furthermore, PCR products of both amplifications (with external and internal primers) were purified using a Wizard SV Gel and PCR Clean-Up System (Promega). DNA concentration of the amplicons was measured with a biophotometer (Eppendorf). PCR products were then digested with restriction enzymes *Ban*I and *Apa*I (Promega) according to the manufacturer's recommendations. Digested DNA was analysed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The results of restriction enzyme analysis, which discriminates between *Mycobacterium marinum*, *M. fortuitum* and *M. chelonae* were interpreted as described by Talaat et al. (1997).

## RESULTS

### Bacteriology

Among 35 ZN-stained specimens, 13 (37.1%) gave positive microscopic results for mycobacteria. Using the

culture method, 29 (82.9%) mycobacterial isolates were obtained as shown in Table 1. Two specimens were ZN-positive but culturing failed because the material was highly contaminated. Four specimens were both ZN- and culture-negative. Identification of the species on the basis of physical and biochemical examination was successfully accomplished for 19 (65.5%) isolates: 7 were identified as *Mycobacterium fortuitum*, 6 as *M. marinum*, 4 as *M. gordonae*, and 2 as *M. chelonae*. However, only 6 isolates completely matched the published data of distinctive biochemical properties of mycobacteria encountered in clinical specimens described by Metchock et al. (1998). All other isolates were identified on the basis of best-fit analysis (Table 1).

### GenoType *Mycobacterium* assay

On the basis of the GTM assay performed on 28 isolates, 23 (82.1%) isolates were identified: 7 as *Mycobacterium fortuitum*, 6 as *M. gordonae*, 6 as *M. marinum*, 3 as *M. chelonae*, and 1 as *M. peregrinum*. The identification of 5 isolates failed due to non-specific banding patterns on the strips.

### PCR-RFLP

All of the isolates subjected to PCR amplification of 16S rRNA with the first set of primers gave the 924 bp PCR products while the amplification with internal primers yielded 300 bp fragments. Since some of the isolates were previously identified with the GTM assay as species not suitable for investigation with the described restriction enzyme analysis (e.g. *Mycobacterium gordonae*, *M. peregrinum*), only 20 isolates were investigated using this method. Identification of the species was successfully accomplished in 17 (85.0%) cases. Three isolates were identified as *M. chelonae*, 5 as *M. fortuitum* and 9 as *M. marinum*.

Table 1. *Mycobacterium* spp. Results obtained with 3 methods for the identification of mycobacteria to species level. Mycobacteria were isolated from 29 aquarium fish in the period 2001–2004. ns: not successful; nd: not done; ZN: Ziehl-Neelsen

| No. | Fish species | Origin | ZN | Biochemistry | GenoType *Mycobacterium* | PCR-RFLP | Final result |
|---|---|---|---|---|---|---|---|
| 1 | Goldfish *Carassius auratus auratus* | Pet shop | – | ns | *M. gordonae* | nd | *M. gordonae* |
| 2 | Goldfish *C. auratus auratus* | Pet shop | + | *M. fortuitum* | *M. fortuitum* | nd | *M. fortuitum* |
| 3 | Goldfish *C. auratus auratus* | Pet shop | – | *M. fortuitum* | *M. fortuitum* | *M. marinum* | *M. fortuitum* + *M. marinum* |
| 4 | Three-spot gourami *Trichogaster trichopterus* | Pet shop | – | ns | *M. fortuitum* | ns | *M. fortuitum* |
| 5 | Guppy *Poecilia reticulata* | Pet shop | – | ns | ns | nd | *Mycobacterium* spp. |
| 6 | Guppy *P. reticulata* | Pet shop | – | ns | ns | ns | *Mycobacterium* spp. |
| 7 | Goldfish *C. auratus auratus* | Pet shop | – | ns | *M. gordonae* | nd | *M. gordonae* |
| 8 | Three-spot gourami *T. trichopterus* | Pet shop | + | *M. fortuitum* | *M. fortuitum* | *M. fortuitum* | *M. fortuitum* |
| 9 | Guppy *P. reticulata* | Pet shop | + | *M. gordonae* | *M. gordonae* | nd | *M. gordonae* |
| 10 | Cichlid fish *Microgeophagus altispinosus* | Pet shop | – | *M. fortuitum* | *M. fortuitum* | *M. fortuitum* | *M. fortuitum* |
| 11 | Cichlid fish *Pseudotropheus lombardoi* | Private owner | – | ns | *M. peregrinum* | nd | *M. peregrinum* |
| 12 | Dwarf gourami *Colisa lalia* | Private owner | + | *M. fortuitum*[a] | nd | *M. fortuitum* | *M. fortuitum* |
| 13 | Goldfish *C. auratus auratus* | Private owner | – | *M. chelonae* | *M. chelonae* | *M. chelonae* | *M. chelonae* |
| 14 | Goldfish *C. auratus auratus* | Private owner | + | ns | ns | nd | *Mycobacterium* spp. |
| 15 | Goldfish *C. auratus auratus* | Private owner | – | *M. fortuitum*[a] | *M. fortuitum* | *M. fortuitum* | *M. fortuitum* |
| 16 | Goldfish *C. auratus auratus* | Private owner | – | ns | ns | ns | *Mycobacterium* spp. |
| 17 | Sterlet *Acipenser ruthenus* | Private owner | – | *M. fortuitum*[a] | *M. fortuitum* | *M. fortuitum* | *M. fortuitum* |
| 18 | Goldfish *C. auratus auratus* | Private owner | + | *M. gordonae*[a] | *M. gordonae* | nd | *M. gordonae* |
| 19 | Goldfish *C. auratus auratus* | Private owner | – | *M. marinum* | *M. marinum* | *M. marinum* | *M. marinum* |
| 20 | Goldfish *C. auratus auratus* | Private owner | + | *M. marinum* | *M. marinum* | *M. marinum* | *M. marinum* |
| 21 | Freshwater angelfish *Pterophyllum scalare* | Pet shop | – | *M. gordonae*[a] | *M. gordonae* | nd | *M. gordonae* |
| 22 | Freshwater angelfish *P. scalare* | Pet shop | – | ns | *M. chelonae* | *M. chelonae* | *M. chelonae* |
| 23 | Freshwater angelfish *P. scalare* | Pet shop | – | *M. gordonae* | *M. gordonae* | *M. marinum*[b] | *M. gordonae* |
| 24 | Freshwater angelfish *P. scalare* | Pet shop | – | ns | ns | *M. marinum*[b] | *Mycobacterium* spp. |
| 25 | Three-spot gourami *T. trichopterus* | Pet shop | + | *M. marinum*[a] | *M. marinum* | *M. marinum* | *M. marinum* |
| 26 | Three-spot gourami *T. trichopterus* | Pet shop | + | *M. chelonae* | *M. chelonae* | *M. chelonae* | *M. chelonae* |
| 27 | Catfish *Corydoras* spp. | Private owner | + | *M. marinum* | *M. marinum* | *M. marinum* | *M. marinum* |
| 28 | Southern platyfish *Xiphophorus maculatus* | Private owner | + | *M. marinum* | *M. marinum* | *M. marinum* | *M. marinum* |
| 29 | Southern platyfish *X. maculatus* | Private owner | – | *M. marinum* | *M. marinum* | *M. marinum* | *M. marinum* |

[a]Biochemical profile completely matching the published data of distinctive biochemical properties of mycobacteria encountered in clinical specimens (Metchock et al. 1998)
[b]Irrelevant results

However, 3 isolates (Nos. 3, 23 and 24, Table 1) identified as *M. marinum* gave different results in other identification tests. Isolate No. 3 was identified as *M. fortuitum* with the GTM assay and with biochemical examination. Based on the colony morphology investigation of the subcultures, it was concluded that the inconsistency of the results was a consequence of mixed infection. Isolate No. 23 was subsequently identified as *M. gordonae* with the GTM assay; the identity was also confirmed with AccuProbe *Mycobacterium gordonae* assay. Isolate No. 24 had the same restriction enzyme profile as isolates Nos. 3 and 23 but it could not be identified with the GTM assay nor with biochemical examination.

## DISCUSSION

The identification of mycobacteria in tissues is traditionally made by ZN staining. However, acid-fast bacilli (AFB) may not always be found through direct microscopy even though granulomatous lesions are present. Our study revealed positive microscopy results in only 37.1% cases while Lescenko et al. (2003) reported a higher proportion of ZN-positive results (58.8%) in fish parenchymatous organs with granulomas. Low AFB detection (9 to 13.2%, respectively) was reported in *Mycobacterium marinum* studies in humans (Edelstein 1994, Ang et al. 2000). The reasons for the failure to identify AFB through direct microscopy may include the destruction of mycobacteria or their low number, as well as the possibility of other microorganisms causing granulomas. *Streptococcus iniae* (Chang & Plumb 1996, Perera et al. 1998, Shoemaker et al. 2000), fungus *Aphanomyces* (Hatai et al. 1994, Wada et al. 1994, Roberts & Rodger 2001) and bacteria of the genus *Nocardia* (Wolke & Meade 1974, Chen 1992) have been isolated from the granulomas of cultured and wild fish. Culture examination is a more sensitive method than direct microscopy. Therefore direct microscopic examination should be considered only as an auxiliary diagnostic method. However, it may provide valuable information especially when culture examination fails. Negative cultivation results may be explained by the killing of mycobacteria caused by host defence mechanisms, a low number of viable mycobacteria in the tissue, or by destruction of the mycobacteria during the preparation of the sample (Lescenko et al. 2003).

Biochemical examination is a time consuming method, the results are often difficult to interprete and variations among strains may occur. In many instances it is necessary to identify the organisms based on a best-fit analysis which may result in erroneous identification (Metchock et al. 1998). Additionally, there are usually small differences in biochemical properties among different mycobacterial species which infect fish. Some species with similar physical characteristics may differ in only one or 2 tests (e.g. *Mycobacterium fortuitum* and *M. chelonae* or *M. marinum* and *M. gordonae*). The reason for the difficult interpretation of the biochemical profile may lie in mixed mycobacterial cultures due to contamination with other bacteria.

Identification of mycobacteria by amplification of a variety of target DNA sequences has been accomplished: 16S rRNA (Rogall et al. 1990, Kox et al. 1995), IS*986* (Kolk et al. 1992), IS*6110* (Noordhoek et al. 1995), and *hsp65* (Plikaytis et al. 1992, Telenti et al. 1993). The possibilities of direct detection and identification of mycobacterial DNA in field samples using molecular methods have been tested with varying success (Talaat et al. 1997, Fiedler et al. 2000). Talaat et al. (1997) described a method that relies on PCR amplification with RFLP of the amplified fragment. This method, discriminating between 3 mycobacterial species, can be applied to either isolated colonies or to infected fish tissues. In the present study, this method was used to identify the isolated colonies. The GenoType *Mycobacterium* (GTM) assay, allowing the identification of 13 species of mycobacteria, was also performed. A comparison of both methods was performed on 19 isolates. The results of the PCR-RFLP were completely consistent with the results of the GTM assay in 15 cases, including 2 cases when both methods failed to identify the species. The lack of an adequate amount of culture, or mycobacteria other than the species recognizable by PCR-RFLP and GTM assay, are possible reasons for the negative outcome of the testing. In one case (isolate No. 4, Table 1) PCR-RFLP failed but the GTM assay identified the isolate as *Mycobacterium fortuitum*. In 3 cases the methods gave inconsistent results (Table 1). For the isolate No. 3 it was concluded that the reason might lie in dual infection. Isolates Nos. 23 and 24 were first tested with PCR-RFLP which revealed the *M. marinum* restriction enzyme profile. Subsequently, the GTM assay was performed and isolate No. 23 was identified as *M. gordonae* while isolate No. 24 could not be identified. Because of identical restriction enzyme profiles of *M. marinum* and *M. gordonae*, the sequence similarity of their 16S rRNA genes was checked. The sequence identity was 97%; the same restriction sites for *Apa*I and no restriction site for *Ban*I were found in both 16S rRNA resulting in the same length of theoretical restriction enzyme fragments. The sequence comparison was made on *M. marinum* strain DSM 44344 and *M. gordonae* strain agha3 (GenBank accession numbers AJ536032 and AJ581472, respectively). Restriction enzyme profiles identical to that of *M. marinum* might also be the case for mycobacteria other than *M. gordonae* which may

lead to false diagnosis. Therefore, this method is probably of little diagnostic value, regardless of the fact that it can be applied directly to infected fish tissues. It enables only the differentiation among a few, albeit the most important species of the mycobacterial species that can infect fish, it is quite time consuming, and most importantly, it may not give reliable results when applied to field samples.

The GTM assay was quite successful; it could be completed within a few hours and allowed the identification of a broad variety of mycobacteria. However, both molecular methods used in the present study may give negative or ambiguous results. To confirm or to further examine unusual results, sequencing of the PCR products or probe-based techniques could be used.

Species of mycobacteria identified in the present study (except *Mycobacterium peregrinum*) are recognized as aquarium fish pathogens. Lescensko et al. (2003) reported the isolation of *M. marinum*, *M. gordonae*, *M. triviale* and *M. avium* subsp. *hominissuis* from 70 aquarium fish in the Czech Republic. However, the latter 2 species were regarded as accompanying microflora. *M. fortuitum*, *M. chelonae* and *M. abscessus* were reported as causative agents of mycobacterial infections in laboratory-maintained zebrafish *Brachydanio rerio* (Astrofsky et al. 2000). *M. fortuitum* has also been isolated from Japanese medaka *Oryzias latipes* (Sanders & Swaim 2001). McCormick et al. (1995) reported *M. chelonae* in cichlid oscar *Astronotus ocellatus*. *M. fortuitum* and *M. smegmatis* were found to be pathogenic for goldfish (Talaat et al. 1999). We have not managed to find any reports describing *M. peregrinum* infection in fish. Therefore, our results might be the first report of the isolation of *M. peregrinum* from an aquarium fish.

Fish mycobacterioses also pose a risk to the human population. People become infected while working with diseased aquarium fish (Street et al. 1991, Antonio et al. 2000, Lehane & Rawlin 2000). The consumption of insufficiently heat-treated fish is a presumed source of potentially pathogenic mycobacteria for immunocompromised patients (von Reyn et al. 1996, Ristola et al. 1999). Many case reports of *Mycobacterium marinum* infections in man exist (Engbaek et al. 1980, Collins et al. 1985, Huminer et al. 1986, Alinovi et al. 1993, Jernigan & Farr 2000, Casal & Casal 2001, Enzensberger et al. 2002, Trampuz et al. 2002, Lewis et al. 2003). Jernigan and Farr (2000) reported that 49.2% of the *M. marinum* infections in humans were aquarium-related and only 2.6% of infections were due to swimming pool-associated injuries. A retrospective study from 1991 to 1998 of *M. marinum* infections in humans in Spain revealed that 35 out of 39 cases were fish related (Casal & Casal 2001). A fish-to-human transmission of mycobacteria other than *M. marinum* is also possible. A recent report by Collina et al. (2002) describes *M. chelonae* and *M. fortuitum* infection in 2 people who kept fish.

*Mycobacterium marinum* infections in humans have been detected in Slovenia. Since 1999, 4 cases were reported by the University Clinic of Respiratory and Allergic Diseases Golnik (M. Žolnir-Dovč unpubl. data). However, *M. marinum* infections in the human population in Slovenia may be more common, but are not diagnosed since there are usually no investigations of such cases. Hand skin lesions were present in all of the 4 affected patients, 3 of whom had a history as aquarium hobbyists.

This stresses the importance of recognizing fish mycobacterioses in order to prevent their transmission to humans.

## CONCLUSIONS

The lack of AFB in ZN-stained smears does not exclude the possibility of fish mycobacterial infection; a complete investigation including culture and molecular methods is required for a reliable diagnosis. Traditional methods for identification of mycobacteria are well established and relatively inexpensive, but are slow in providing clinically relevant information and are limited in scope to the species for which a large number of strains have been studied. The best diagnostic option to identify the species of mycobacteria from cultured material is the GenoType *Mycobacterium* assay since it enables fast detection of a variety of mycobacterial species. Since we have detected *Mycobacterium marinum* in the investigated fish and since Slovenia experiences *M. marinum* infections in humans every year, the importance of mycobacterial infections in aquarium fish should not be overlooked in veterinary or in human medicine.

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