

## Mycobacterial infection in aquarium fish

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**ABSTRACT:** In the first part of the study, 70 aquarium fish of different species, originating from stocks from the Czech Republic, were pathomorphologically examined for the presence of granulomatous lesions. Granulomas were diagnosed in 44 (62.9%) fish. From these, acid fast rods (AFR) were found in 29 (65.9%) by staining according to Ziehl-Neelsen (Z-N). In the remaining 26 (37.1%) fish without granulomatous lesions, the presence of AFR was microscopically proven in 3 (11.5%) fish. In the second part of the study, direct microscopic examinations according to Z-N and culture examinations for the presence of mycobacteria were carried out in 17 randomly selected fish of the *Apistogramma cacatuoides*, *Trichogaster leeri*, *Trichogaster trichopterus*, *Pterophyllum scalare*, *Paracheirodon axelrodi* species with the pathomorphological discovery of granulomas. Mycobacteria were culturally found in 12 (70.6%) fish. *Mycobacterium marinum*, pathogenic for fish, was isolated from 6 fish, of which in one *M. triviale* was found at the same time and in two *M. avium* mixed serotypes 6, 8 and 9 (genotype IS901–, IS1245+). *M. gordonae* was isolated from a further 6 fish, of which in two *M. avium* mixed serotypes 6, 8 and 9 (genotype IS901–, IS1245+) was also isolated at the same time. A mixed infection of more species of mycobacteria was therefore found in five fish. In these it may be assumed that the mycobacteria of the *M. triviale* and *M. avium* mixed serotypes 6, 8 and 9 (genotype IS901–, IS1245+), non-pathogenic for fish, came from the water environment and “merely contaminated” the tissues of the fish.

**Keywords:** mycobacteriosis; granulomas, serotyping

The mycobacterial infection of freshwater fish was first described as early as 1897 (Bataillon *et al.*, 1897). In areas of the temperate zone and in the tropics, mycobacterial infection is among the most common chronic diseases of freshwater and sea fish (Roberts and Schlotfeldt, 1985; Noga, 1995; Kiesch, 2000). In the last few years, mycobacteriosis has been described in many species of freshwater and sea fish in the wild and kept in captivity, in which it causes major economic losses.

Chronic infections caused by different species of mycobacteria have been found in fish from the Mediterranean Sea [sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), silver mullet (*Mugil curema*) and amberjack (*Seriola dumerili*)] (Rodgers and Furones, 1998). Mycobacterial infections were also found in sea fish in Japan, causing economic losses

(Kusuda and Kawai, 1998). There was a spontaneous outbreak of mycobacteriosis in fancy veiltail guppies (*Lebistes reticulatus*), raised on an ornamental fish farm in Venezuela. Numerous mycobacteria were detected in smears from the kidneys, liver, mesentery and spleen of the fish and from fresh faecal material from clinical ill fish with listlessness, emaciation, spinal curvature, sunken eyes and loss of colour (Conroy and Conroy, 1999).

Mycobacteriosis was found in two stocks of zebrafish (*Brachydanio rerio*) in the presence of symptoms like skin erosions and ulceration along the flank just caudal to the pectoral fins, hyperemic gills, petechia around the opercula, abdominal distention, emaciation, congested liver with inflammation and multifocal hepatic necrosis (Astrofsky *et al.*, 2000). Antonio *et al.* (2000) found mycobacte-

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riosis in fish of the delta smelt species (*Hypomesus transpacificus*) in the wild and kept in captivity at a time when they were subject to different stresses (a water temperature higher than 17°C or the period of spawning). Mycobacteriosis was diagnosed in wild rabbitfish (*Siganus rivulatus*) populations associated with commercial mariculture cages and inhabiting various sites along the Israeli Red Sea coastline (Diamant *et al.*, 2000).

Ingestion of mycobacteria is probably the major source of infection, including fish that have recently eaten dead tankmates (Noga, 1995). Another possible means of mycobacterial infection is through injured skin, to which numerous identifications of mycobacteria in skin lesions bear witness (Inglis *et al.*, 1993; Astrofsky *et al.*, 2000; Heckert *et al.*, 2001; Herbst *et al.*, 2001). Given that the mycobacteria were isolated from unborn embryos from infected viviparous mothers, Conroy and Conroy (1999) posit the transovarial transmission of mycobacteria in viviparous fish.

Many different species of mycobacteria belonging to both fast and slow growing species have been isolated from fish (Wayne and Kubica, 1986). However, those most frequently isolated were mycobacteria of the species *Mycobacterium marinum*, *M. fortuitum*, *M. chelonae* and *M. avium*, which are considered pathogenic for fish (Thoen and Schliesser, 1984; Stoskopf, 1993; Noga, 1995; Sanders and Swaim, 2001). Of the other species of mycobacteria, the following have been isolated: *M. aurum*, *M. gordonae*, *M. parafortuitum*, *M. poriferae* a *M. triplex* (Arakawa and Fryer, 1984; Arakawa *et al.*, 1986; Bragg *et al.*, 1990; Dalsgaard *et al.*, 1992; Stoskopf, 1993; Tortoli *et al.*, 1996; Teska *et al.*, 1997; Bruno *et al.*, 1998; Talaat *et al.*, 1999; Diamant *et al.*, 2000; Herbst *et al.*, 2001; Perez *et al.*, 2001). In certain species of mycobacteria, their pathognomic importance has not yet been made clear. For example, *M. chelonae* subsp. *abscessus* was isolated from 25 to 100% of fish examined of the Japanese medaka species (*Oryzias latipes*) at the age of 2 to 27 months, without finding clinical symptoms of mycobacteriosis or a significantly higher mortality (Teska *et al.*, 1997).

The diagnosis of mycobacterial disease in fish is possible on the basis of the discovery of granulomas during autopsy in native preparates or during histopathological examination. Mycobacteria are identified by staining the smears according to Ziehl-Neelsen (Z-N) as acid fast-rods (AFR), which occur independently or in clusters (Daoust *et al.*,

1989; Diamant *et al.*, 2000). Immunohistochemical methods have been used in the diagnosis of mycobacteriosis in many studies (DeMartinez and Richards, 1991; Dalsgaard *et al.*, 1992; Hatai *et al.*, 1993; Gomez *et al.*, 1996; Teska *et al.*, 1997; Colorni *et al.*, 1998; Bruno *et al.*, 1998; Conroy and Conroy, 1999; Wolf and Smith, 1999; Astrofsky *et al.*, 2000; Sanders and Swaim, 2001). Culture examination is appropriate for use not only to make previous diagnostic methods more precise, but also to obtain a mycobacterial isolate which may be more closely defined e.g. biochemically or using molecular biological methods (Wayne and Kubica, 1986; Rychlik and Pavlik, 1997; Matlova *et al.*, 1998; Bruno *et al.*, 1998; Dvorska *et al.*, 1999a, 2001; Antonio *et al.*, 2000; Heckert *et al.*, 2001; Herbst *et al.*, 2001; Ucko *et al.*, 2002). The possibilities of direct detection and identification of the DNA of mycobacteria in biological material using methods of molecular biology have also been tested with varying success in the diagnosis of mycobacterioses (Talaat *et al.*, 1997; Fiedler *et al.*, 2000).

Mycobacteriosis in fish is also a very frequently laboratorially diagnosed disease in the Czech Republic (Navratil *et al.*, 2000). For this reason, in the first part of our work, the frequency of the incidence of granulomas and AFR during the routine pathomorphological examination of aquarium fish was monitored. In the second part, the direct microscopic examination according to Z-N of a further 18 randomly selected fish in which granulomas had been found was supplemented by a cultivation examination for mycobacteria with subsequent species identification of isolates.

## MATERIAL AND METHODS

### The first phase of the work

In the first phase of our work, a total of 70 aquarium fish which had been delivered for examination from stocks in the Czech Republic with different health problems were examined (Table 1). Their bodies were subjected to pathomorphological and microscopic examination of native preparates with a focus on granulomatous lesions in the skin and in parenchymatous organs. The wet mounts were subsequently stained according to Z-N to identify AFR. Skin scrapings and prints of the skeletal musculature and individual organs of the body cavity (hepatopancreas, spleen, kidneys and contents of the intestine) from each fish were examined.

Table 1. Pathomorphology and Ziehl-Neelsen staining (first part of the study)

PM examination			Z-N staining		
Lesions	No.	%	AFR	No.	%
–	26	37.1	–	23	32.9
			+	3	4.3
+	44	62.9	–	15	21.4
			+	29	35.8
Subtotal			–	38	54.3
			+	32	45.7
Total	70	100		70	100

PM = pathomorphology (findings of granulomas) in parenchymatous organs

Z-N = Ziehl-Neelsen staining

AFR = acid fast rods after the Z-N staining

method and cultured at temperatures of 25°C and 37°C on three media (one egg yolk medium according to Herrold, one liquid serum medium according to Sula and one egg medium according to Stonebrink). The growth of mycobacterial strains was monitored every 2 weeks for a period of two months (Fischer *et al.*, 2000).

The mycobacterial strains obtained were identified according to their morphological appearance, by staining according to Z-N and using biochemical methods (Wayne and Kubica, 1986). The PCR method with primers for detecting IS901 (Kunze *et al.*, 1992), IS1245 (Guerrero *et al.*, 1995) and serotyping described by Wolinsky and Schaefer (1973) and modified by Süssland and Hrdinova (1976) were used to identify isolates of the *M. avium* complex.

## RESULTS

### The first phase of the work

In the first part of the study, of the 70 fish examined, pathomorphological changes were found in 44 (62.9%). AFR were identified in the parenchymatous organs in 29 fish in which granulomas were discovered and in 3 of 26 fish without granulomas. In total, AFR were therefore found in 32 (45.7%) of the 70 fish examined (Table 1).

### The second phase of the work

In the second phase, a further 17 randomly selected individuals from five species of aquarium fish with granulomas in parenchymatous organs were culturally examined (Table 2). Samples of tissue for culture examination were homogenised in a mortar, then centrifuged, treated using the HCl-NaOH

Table 2. Mycobacteria detection by culture examination in parenchymatous organs with granulomas (second part of the study)

Z-N staining			Culture examination		
AFR	No.	%	isolation	No.	%
–	7	41.2	–	4	57.1
			+	3*	42.9
+	8	47.0	–	1	12.5
			+	7	87.5
++	2	11.8	–	0	0
			+	2	100
Subtotal			–	5	29.4
			+	12	70.6
Total	17	100		17	100

Z-N = Ziehl-Neelsen staining

AFR = acid fast rods after the Z-N staining (– not detected, + detected in low concentration, ++ detected in massive amounts)

\*fish No. 1, 5 and 7 from Table 3

Table 3. Mycobacterial species isolated from fish with granulomas

Fish species	Fish No.	<i>M. marinum</i>	<i>M. triviale</i>	<i>M. gordonae</i>	<i>M. avium</i> *
Cacatoo Dwarf Cichlid ( <i>Apistogramma cacatuoides</i> )	1	+	+	–	
	2	–	–	+	+ serotype 8
	3	+	–	–	+ serotype 9
	4	+	–	–	+ serotype 9
	5	+	–	–	–
	6	–	–	+	–
	7	+	–	–	–
	8	–	–	+	+ serotype 6
Pearl Gourami ( <i>Trichogaster leeri</i> )	9	+	–	–	–
Three Spot Gourami ( <i>Trichogaster trichopterus</i> )	10	–	–	+	–
Angelfish ( <i>Pterophyllum scalare</i> )	11	–	–	+	–
Cardinal Tetra ( <i>Paracheirodon axelrodi</i> )	12	–	–	+	–

*M.* = *Mycobacterium*, \**M. avium* subsp. *hominissuis* isolates of genotype IS901– and IS1245+ (Mijls *et al.*, 2002)

## The second phase of the work

In the 17 randomly selected fish in which granulomas were found AFR of differing intensity were identified in 10 fish, of which mycobacteria were culturally isolated in 9 of them. After the examination of seven fish without direct microscopic identification of AFR, the culture examination was positive in three further fish (Table 2).

From the total of 12 culturally positive fish (70.6%), four species of mycobacteria were isolated: *M. marinum*, *M. gordonae*, *M. triviale* and *M. avium* subsp. *hominissuis* genotype IS901– and IS1245+ and serotypes 6 ( $n = 1$ ), 8 ( $n = 1$ ) and 9 ( $n = 2$ ) (Mijls *et al.*, 2002). In five fish, two species of different mycobacteria were identified (Table 3). *M. marinum* was isolated (Table 3) from three fish without direct microscopic identification of AFR in granulomatous lesions (Table 2).

## DISCUSSION

### The first phase of the work

From the identification of granulomatous lesions in more than 60% of the fish examined originating from stocks of aquarium fish in the Czech Republic with health problems, it is possible to draw conclusions about the high frequency of chronic infections accompanying the creation of granulomas. Since AFR were not found in all granulomatous lesions

through direct microscopy (Table 1), it is possible to presume infection through other sources causing the creation of granulomas. Wolke and Meade (1974) and Chen (1992) have described the creation of granulomas after infection with bacteria of the genus *Nocardia*. Chang and Plumb (1996), Perera *et al.* (1998) and Shoemaker *et al.* (2000) isolated *Streptococcus iniae* from the granulomas of fish. Dykova *et al.* (1996) identified amoeba in granulomatous lesions and Hatai *et al.* (1994), Wada *et al.* (1994), Vishwanath *et al.* (1998) and Roberts and Rodger (2001) identified fungi (notably species *Ichthyophonus hoferi* and *Aphanomyces invadans*). Another reason for the failure to identify AFR in granulomatously changed tissue through direct microscopy could have been the destruction of mycobacteria, or their low number, which was microscopically undetectable. Similar discoveries have also been described in other species of animals with granulomatous infection, e.g. in pigs. In these it was found that not only mycobacteria, but also bacteria of the *Rhodococcus equi* species contributed to the emergence of tuberculous lesions in the cephalic and intestinal lymph nodes (Dvorska *et al.*, 1999b).

The reverse finding was made in three fish in which pathomorphological changes of a granulom character were not found, but in which AFR were found through direct microscopy (Table 1). The cause was probably the initial phase of the pathomorphological process before the creation of visible granulomas in the parenchymatous organs.

It may also have been a question of contamination of the body surface, branchiae or digestive tract of aquarium fish with non-pathogenic mycobacteria from the water environment, in which mycobacteria often occur (Horvathova *et al.*, 1997; Kazda, 2000).

### The second phase of the work

In the second phase of our work, by comparing the results of direct microscopic and culture examination for the presence of AFR, we had a higher yield from culture examination (Table 2). The pathomorphological examination of fish supplemented by direct microscopic examination for the detection of AFR may therefore be considered as a fast, but only auxiliary examination. This is indicated by the culture identification of mycobacteria pathogenic for fish in three individuals nos. 1, 5 and 7 (Table 3) without microscopic identification of AFR (Table 2). The negative cultivation results in one fish with microscopically identified AFR (Table 2) may be explained by a devitalisation of mycobacteria through the defence mechanisms of the host organism. Nor may other factors be ruled out, such as, for example, a low number of live mycobacteria in the tissue examined, which may have been killed during the preparation of the sample. It is also known that nocardia, which stain like AFR, may cause infection in fish accompanied by the creation of a granulomatous infection (Wolke and Meade, 1974; Wayne and Kubica, 1986; Chen, 1992).

It therefore follows from these results that cultivation examination may supplement and above all make more precise the routine diagnosis of mycobacterial infections in fish based only on pathomorphological examination and direct identification of AFR after staining the prepareate according to Z-N.

On the basis of biochemical methods, it was found that in the fish with granulomas which we examined, two species of mycobacteria occurred: *M. marinum* and *M. gordonae* (Table 3). The acceleration of the course of the disease in infected individuals may be assisted by the weakening of their immune system, leading to a generalisation of the infection (Wayne and Kubica, 1986; Noga, 1995; Matlova *et al.*, 1998; Antonio *et al.*, 2000). However, other species of mycobacteria (*M. triviale* a *M. avium* subsp. *hominissuis* of serotypes 6, 8 a 9 and genotypes IS901– and IS1245+) were also isolated from the fish tissue examined. These occur normally in

the water and external environment and are considered non-pathogenic species for fish (Arakawa *et al.*, 1986; Wayne and Kubica, 1986; Matlova *et al.*, 1998). Mycobacteria pathogenic and non-pathogenic for fish may be brought into an aquarium together with aquarium plants, sand, water or through the contaminated surface of various aquarium appliances (Goslee and Wolinsky, 1976; Horvathova *et al.*, 1997; Pavlik *et al.*, 2000; Kiesch, 2000; Kazda, 2000).

From a pathogenic perspective, it is also important to note that the mycobacteria did not occur in the afflicted fish independently, but were always found together with other species of mycobacteria pathogenic for fish (Table 3). It may therefore be assumed that they were not the main cause of disease in the fish, but may have appeared with the weakening for the organism or simply through accompanying mycobacterial “contamination”.

Mycobacterial infections in fish also present a risk factor for the human population. They become infected while working with the contents of aquaria with infected fish (Street *et al.*, 1991; Vincenzi *et al.*, 1992; Shih *et al.*, 1997; Smith, 1997; Speight and Williams, 1997; Lehane and Rawlin, 2000; Antonio *et al.*, 2000). The consumption of insufficiently heat treated fish foods is a presumed source of potentially pathogenic mycobacteria for patients infected with HIV (Von Reyn *et al.*, 1996; Ristola *et al.*, 1999). For example, *M. marinum* has been isolated from patients with skin lesions and infected lymph nodes (Alinovi *et al.*, 1993; Edelstein, 1994; Ang *et al.*, 2000; Jernigan and Farr, 2000; Seiberras *et al.*, 2000; Bhatti *et al.*, 2000), which has also been identified in infected fish in the Czech Republic (Table 3).

### CONCLUSIONS

The following conclusions may be drawn from our results:

1. The incidence of mycobacterial infections in fish may be considered relatively high in the Czech Republic.
2. The examination of fish focusing on mycobacterial infections which is based only on pathomorphological examination and direct microscopic identification of AFR according to Z-N is inadequate for making a final diagnosis.
3. The culture identification of mycobacteria without identifying the isolate cannot lead to the discovery of the etiological agent of fish disease because of the incidence of atypical mycobacteria in the water environment of the aquarium.



4. The species of mycobacteria isolated in our study are potentially pathogenic mycobacteria for the human being. Infected aquarium fish in the Czech Republic therefore also represent a risk factor for the human population.

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