Detection of Mycobacteria in Ornamental Fish in Iran by Culture and Ziehl-Neelsen Staining Methods

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ABSTRACT

Background: Mycobacterial infections in fish is largely chronic to subacute in nature and affects fishes in fresh water, brackish water and salt water. In addition to their known infectivity to fishes, aquatic mycobacteria pose significant zoonotic concerns. Due to the zoonotic character of the disease, increasing importance of aquariology, and lack of any clinical signs in early steps of mycobacteriosis, present study was undertaken to analyze the distribution of mycobacteria in diseased and apparently healthy ornamental fish from some local aquarium fish shops in four different cities in Iran by culture and Ziehl-Neelsen staining.

Materials, Methods & Results: One hundred and one fresh water aquarium fish of 22 species from some local shops in four cities in Iran were examined. Before decontamination, smears of homogenized samples stained with Ziehl-Neelsen. For bacterial culture, samples were inoculated on Lowenstein-Jensen medium. Culture plates were examined daily for four weeks. The rate of growth at different temperature, colony morphology and pigmentation were evaluated for species identification. Among 79 moribund fish examined, 16 individuals were positive for acid fast rods at microscopic examination. Seven fish out of 22 apparently healthy individuals also gave positive microscopic results. Using the culture method, 29 and 10 Mycobacterium isolates were obtained from moribund and healthy fish. The following Mycobacterium species were isolated from unhealthy fish: Mycobacterium fortuitum, M. marinum, and M. smegmatis. The number of different species of Mycobacterium from apparently healthy fish was: M. fortuitum (one fish), M. marinum (one fish), M. terrae (one fish) and M. flavescens (one fish).

Discussion: Based on the results in both moribund and apparently healthy fish examined, culture examination showed more mycobacteria than Ziehl-Neelsen staining detection. Lower proportion of Ziehl-Neelsen positive results compared with culture method was reported in aquarium fish in Slovenia. Using positive microscopic results, 13 isolates were obtained where as 29 samples gave positive culture results for mycobacteria. Similar result was also observed in clinically healthy ornamental fish and their aquarium environment. The identification of mycobacteria by Ziehl-Neelsen staining is a traditional method. However, acid-fast bacilli may not always be found through direct microscopy because the destruction of mycobacteria or their low number may sometimes happen. Culture examination is a more sensitive method than direct microscopy. However, killing of mycobacteria caused by host defense mechanisms, a low number of viable mycobacteria in the tissue, or by destruction of the mycobacteria during the preparation of the sample could result in negative cultivation results. Species of Mycobacterium identified in unhealthy fish were M. fortuitum, M. marinum and M. smegmatis. High frequency of identifying M. fortuitum and M. marinum (6 out of 7) in the samples provide more evidence that these species are common Mycobacterium species to be found in diseased aquarium fish. Numerous studies showed the common isolation of these two species from aquarium fish. M. marinum infection may be an occupational hazard for certain professionals such as pet shop workers. Many infections may also occur in fish fanciers who keep an aquarium at home. Less common than M. marinum, M. fortuitum is also capable of infecting human. In this study, the occurrence of M. marinum and M. fortuitum in both unhealthy and apparently healthy aquarium fish shows the importance of recognizing fish mycobacteriosis in order to prevent their transmission to human.

Keywords: mycobacteriosis, aquarium fish, Iran, Ziehl-Neelsen staining, bacterial culture.
INTRODUCTION

Mycobacterial infections in fish is largely chronic to subacute in nature and affects fishes in fresh water, brackish water and salt water. Until recently, three main species of mycobacteria have been associated with mycobacteriosis in fish, namely Mycobacterium marinum, M. fortuitum and M. chelonae, although several other Mycobacterium species, including a number of new species, have now been associated with mycobacteriosis [7]. Affected fish show variable clinical signs, depending on the main sites of the infection and its severity [6]. Infected fish may appear normal and infection may not be suspected until fish coloration fades and movements become sluggish [6].

In addition to their known infectivity to fishes, aquatic mycobacteria pose significant zoonotic concerns. Certain Mycobacterium species can cause contagious skin infections in humans (under particular conditions) transmitted from aquaria (so-called ‘fish tank granuloma’) or from swimming pools (swimming pool granuloma). These infections are difficult to diagnose in humans and therapy is often complicated [1].

The diagnosis of mycobacterial disease in fish is often based on histopathological, culture and molecular methods. Due to the zoonotic character of the disease, increasing importance of aquariology, and lack of any clinical signs in early steps of mycobacteriosis, present study was undertaken to analyze the distribution of mycobacteria in diseased and apparently healthy ornamental fish from some local aquarium fish shops in four different cities in Iran by culture and Ziehl-Neelsen staining.

MATERIALS AND METHODS

Fish

From April 2010 to September 2010, 101 fresh water aquarium fish of 22 species from some local shops in four cities in Iran (including Tehran, Tabriz, Zanjan, and Shahindezh) were examined. Among 101 fish, 22 samples were with no clinical signs of diseases. Seventy nine moribund fish with clinical sings of anorexia, listlessness, blindness, epithelial lesions, distended abdomen, and deformity of gills were also selected. Live fish were transported in oxygenated insulated coolers to the research center for TB and pulmonary disease of Tabriz University of medical sciences. The live fish were killed by immersion in clove oil (50 µL L^{-1}) and examined immediately.

Laboratory examination

Fish were dissected with sterile instruments. Excised organs, consisted of liver, kidney, spleen, heart, intestine, ovary, brain and eye and whole fish (minus organs) were put into sterile polythene bags and homogenized by a laboratory blender. Before decontamination, smears of homogenized samples were prepared and stained with Ziehl-Neelsen for detection of acid-fast rods. At least 100 fields for each sample were viewed at 1000 magnification.

For bacterial culture, samples were centrifuged and decontaminated with HCl-NaOH. Briefly, the homogenate was exposed to 1% NaOH (w/v) and after shaking for 30 min at room temperature, centrifugation at 3000 g for 30 min was performed. The supernatant was removed and the pellets were resuspended in Butterfield’s phosphate-buffered saline containing the pH indicator 0.01 % phenolphthalein, and neutralized by adding 1% HCl till the elimination of a red color (pH 6.8). Twenty-five microlitres of each homogenate were inoculated on Lowenstein-Jensen medium. For each fish sample, two culture plates were incubated at 30 ± 1°C and 37 ± 1°C. Culture plates were examined daily for 4 weeks and then weekly for up to 3 months [4].

Identification of mycobacterial isolates

Twelve isolates were randomly subjected to physical and biochemical examinations to identify the species. The rate of growth at different temperature, colony morphology and pigmentation were evaluated. Biochemical methods namely nitrate reduction, catalase heat stable 68°C, tween-80 hydrolysis, arylsulphatase activity (three days), urease and niacin production were also performed.

RESULTS

Among 79 moribund fish examined, 16 individuals were positive for acid-fast rods at microscopic examination. Seven fish out of 22 apparently healthy individuals also gave positive microscopic results for species of Mycobacterium. Using the culture method, 29 and 10 mycobacterial isolates were obtained from moribund and healthy fish, respectively.

Twelve isolates were randomly sampled for identification of Mycobacterium species on the basis of physical and biochemical examinations. The following Mycobacterium species were isolated from unhealthy fish: Mycobacterium fortuitum (three fish), M. marinum (three fish), and M. smegmatis (one fish). The number of different species of Mycobacterium from apparently healthy fish was: M. fortuitum (1 fish), M. marinum (one fish), M. terrae (one fish), and M. flavescens (one fish) [Table 1].

Table 1. Biochemical profiles of 12 *Mycobacterium* isolates from fresh water aquarium fish of local shops of four cities in Iran from April 2010 to September 2010.

<table>
<thead>
<tr>
<th>Phenotypic tests</th>
<th><em>M. fortuitum</em></th>
<th><em>M. smegmatis</em></th>
<th><em>M. asiaticum</em></th>
<th><em>M. fl avescens</em></th>
<th><em>M. terrae</em></th>
<th><em>M. marnum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow growers (&gt;7 days)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rapid growers (&lt;7 days)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 30°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 37°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Pigment production</td>
<td>N</td>
<td>N</td>
<td>P</td>
<td>Sc</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Catalase heat stable 68°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tween-80 hydrolysis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arylsulphatase activity (3 days)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Niacin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

P= Photochromogenic; Sc= Scotochromogenic; N= Non-chromogenic; R= Rough; S= smooth. A plus sign (+) indicates that the isolate was positive for the test; a negative sign (-) indicates a negative reaction for the test.

**DISCUSSION**

Based on the results in both moribund and apparently healthy fish examined, culture examination showed more mycobacteria than Ziehl-Neelsen staining detection. Lower proportion of Ziehl-Neelsen positive results compared with culture method was reported in aquarium fish in Slovenia [6]. Using positive microscopic results, 13 isolates were obtained where as 29 samples gave positive culture results for mycobacteria. Similar result was also observed in clinically healthy ornamental fish and their aquarium environment [1], where Ziehl-Neelsen staining was positive in 16 and mycobacteria were detected by culture in 49 samples. The identification of *Mycobacterium* by Ziehl-Neelsen staining is a traditional method. However, acid-fast bacilli may not always be found through direct microscopy because the destruction of mycobacteria or their low number may sometimes happen [6]. Culture examination is a more sensitive method than direct microscopy. However, killing of mycobacteria caused by host defense mechanisms, a low number of viable mycobacteria in the tissue, or by destruction of the mycobacteria during the preparation of the sample could result in negative cultivation results [6].

Identification of *Mycobacterium* species by biochemical examination has been widely accomplished. Results in this time consuming method are often difficult to be interpreted and variations among strains may occur [6]. Species of *Mycobacterium* identified in unhealthy fish were *Mycobacterium fortuitum*, *M. marinum* and *M. smegmatis*. High frequency of identifying *M. fortuitum* and *M. marinum* (6 out of 7) in the samples provide more evidence that these species are common *Mycobacterium* species to be found in diseased aquarium fish. Numerous studies showed the common isolation of these two species from aquarium fish [1,5-7,9]. In this study, less frequently isolated species included *M. smegmatis* (1 out of 7). Talaat et al. [8] also reported *M. smegmatis* to be pathogenic for goldfish.

In apparently healthy ornamental fish, *Mycobacterium* isolates were *M. fortuitum*, *M. marinum*, *M. terrae* and *M. fl avescens*. Each species were isolated from one separate fish sample. Few studies have looked for presence of *Mycobacterium* species in clinically healthy aquarium fish. Beran et al. [1] showed the incidence of 3 species namely *M. fortuitum*, *M. terrae* and *M. gordonae* in some aquarium fish. Since in our study, apparently ornamental fish were selected from the same shops where diseased individuals were samples, healthy fish might be in initial or latent stages or the fish tissues might be merely colonized with mycobacteria present in the aquarium. In previous study, it was assumed that *Mycobacterium* species from aquarium environments may serve as a possible source of infection for aquarium fish [1].

CONCLUSION

Fish mycobacteriosis can pose a risk to the human population. People become infected by direct injury from the fish fins or bites during the handling of the aquariums such as cleaning or changing the water. In fact, *Mycobacterium marinum* infection may be an occupational hazard for certain professionals such as pet shop workers. Many infections may also occur in fish fanciers who keep an aquarium at home [2,3,6]. Less common than *M. marinum*, *M. fortuitum* is also capable of infecting human. In this study, the occurrence of *M. marinum* and *M. fortuitum* in both unhealthy and apparently healthy aquarium fish shows the importance of recognizing fish mycobacteriosis in order to prevent their transmission to human.

SOURCES AND MANUFACTURERS
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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES