

Isolation of *Mycobacterium fortuitum* from fish tanks in Alborz, Iran

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ABSTRACT

Introduction: Fish mycobacteriosis is caused by the non-tuberculous mycobacteria. Infected fish are normally the primary source of infection, although non-tuberculous Mycobacteria can be found in the environment. The present study was designed to investigate the few recently found suspected cases of mycobacteriosis in Iranian ornamental fish tanks.

Materials and Methods: Pathological specimens including granulomas from autopsied fish were used to inoculate Lowenstein-Jensen medium. Genomic material was extracted from all acid-fast positive cultures. The mycobacterial identity of bacterial isolates was authenticated using a PCR assessment targeting a 543 bp-long stretch of 16Sr RNA gene. Further more, a PCR assessment targeting a 294 bp-long stretch of heat shock protein hsp65 was performed and the amplicons were sequenced to identify the isolates.

Results: Characteristic mycobacterial bacilli were identified both in light and fluorescent microscopy of bacterial culture from all the suspected specimens. PCR-amplification of DNA templates from all isolates successfully resulted in production of the expected products. Existence of *Mycobacterium fortuitum* was confirmed by comparison analysis of nucleotide sequencing at hsp65 gene.

Conclusion: The present work clearly shows mycobacteria are important in pathology of ornamental fish diseases. People who are keeping fish as pet in their homes should be cautioned about the bacterial contamination risks arise from close contact with exotic ornamental species of fish.

Keywords: Ornamental fishes, Mycobacteriosis, *Mycobacterium fortuitum*, Granulomatosis

INTRODUCTION

Mycobacterial diseases are among the most common chronic diseases of bacterial etiology found in fish worldwide (1, 2). Laboratory confirmation of mycobacteriosis is based on acid-fast microscopy of suspected pathological material. There is no treatment for fish infected with mycobacteria, so depopulation of an infected group is strongly

recommended. Fish mycobacteriosis is often chronic with limited mortality, but in certain circumstances infections may be acute and result in severe loss in cultured fish (3).

Mycobacteria in aquatics are natural pathogens of ectotherms, including frogs and fish, and their optimal growth temperature range is 25–35°C (4). Mycobacteriosis is a common chronic infection of fish and its prevalence may reach as high as 15% in some fish populations (2). Infected fish exhibit clinical signs including loss of scales, depigmentation, hyperpigmentation, abnormal behavior such as remaining alone in one corner of the aquarium and lethargy. Infected fish also may display a slightly concave ventral surface as a sequel to cachexia (2).

Mycobacteriosis is generally characterized as a

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systemic disease affecting several organ systems. The predominant pathological hallmarks of the infection are the infiltration of lymphoid cells and macrophages with granuloma formation. Chronic inflammatory cells, characterized by having desmosomes with tonofilaments, are found in conjunction with epithelioid cells (2).

Fish mycobacteriosis was first described in carp (*Cyprinus carpio*) from water that contaminated with *Mycobacterium tuberculosis* (5).

Mycobacterium marinum was firstly isolated from saltwater fish in the Philadelphia aquarium (6), *M. fortuitum* was initially isolated from the neon tetra (*Paracheirodon innesi*) in 1953 (7) and *M. chelonae* was found in Chinook salmon by Ashburner (*Oncorhynchus tshawytscha*) (8).

Several mycobacterium including *M. abscessus*, *M. avium*, *M. chelonae*, *M. chesapeaki*, *M. fortuitum*, *M. gordonae*, *M. homophile*, *M. lentiflavum-like*, *M. marinum*, *M. montefiorensis*, *M. montefiorensis-like*, *M. peregrinum/septicum*, *M. neoaurum*, *M. pseudoshottsii* and *Mycobacterium chelonae* are the etiological agents of fish Mycobacteriosis. *Mycobacterium scrofulaceum*, *M. shottsii*, *M. simiae*, *M. triplex-like* and *M. szulgai* have been identified from different fish species (5).

Three Mycobacterial species namely *M. marinum*, *M. fortuitum* and *M. chelonae* have dominated the literature on fish diseases and have been considered as potential risks for human. These species can cause contagious skin infections in human and cause medical conditions known as fish tank granuloma and swimming pools granuloma (1, 5, 9 and 10). Infection in human consists of nodular cutaneous lesions that can progress to tenosynovitis, arthritis, and osteomyelitis, depending on the immunological status. The number of human infections caused by *M. fortuitum* is on the rise (11).

The purpose of this study was therefore identification and characterization of mycobacterial isolates recently collected from fish tank in Karaj city, Iran.

MATERIALS AND METHODS

Sampling. The sum 50 different samples of ornamental fish were selected from four different aquariums during June 2011- June 2012. These frozen samples were chosen randomly from aquariums and transferred to the laboratory of Tuberculosis Department at Razi Vaccine & Serum Research

Institute, Karaj, Iran.

At RVSRI, all fish were autopsied in a microbiological cabinet where an individually set of sterile instruments was used for each animal. In brief, all organs in the abdomen cavity were taken out, cut in to 3 parts (head, body and tail). Then in the sterile plates, each part was cut into small particles and were transmitted to porcelain mortar and grounded. To prevent secondary infection growth, NaOH was added to the samples obtained from highly contaminated organ such and for some tissue with low contamination, mixture of NaOH, Sodium nitrate and N-acetyl-L-cysteine was added to the samples of head and tails (12). All samples were kept at the room temperature as intestine for 15-20 minutes (Fig. 1).

Then 5-10 ml supernatant was transmitted to the sterile falcon tube and 40-45 ml phosphate tampon without phenol was added. To neutralize the pH of sample, 1-2 drops of Bromothymol blue with 5 ml normal HCL were added to the samples that were disinfected with NaOH.

Then falcon tubes about 15 minutes were centrifuged in 3000 g and 0.1 ml of sediments inoculated to each media (Lowenstein-Jenson with pyruvate (LJP) (12), Lowenstein-Jenson with glycerol (LJG), herrold egg without and with mycobactin, and MGIT (Mycobacterium growth Indicator tube) and then cultured tubes kept at 25°C. To all MGIT tubes, PANTA antibiotic and OADC as enrichment were added. PNATA included Polymyxin B, Amphotericin, Nalidixic acid, Trimethoprim and Azolusin that prevented growth of other bacteria except mycobacterium (13) (Fig. 3). At the same time from the sediments of each sample, two smears were prepared and stained with ziehl-Neelsen and fluorochrome and observed under light and fluorescence microscope respectively.

The genomic DNA for PCR was extracted from the visible colonies according to the method described by van Embden *et al* (1992) as below (14). At least one loop full of *Mycobacterial* growth was, placed into 400 µl of 1X TE buffer (10mM Tris/HCl, 1 mM EDTA, pH= 8.0), and incubated at 80°C for 20 min to inactivate the cells. 50 µl of lysozyme (10 mg/ml), was added to the heat killed cells and the suspension was vortexed shortly and incubated overnight. It followed by addition of 75 µl of proteinase K (10mg/ml) in 10% SDS. The suspension was vortexed and incubated for 10 min. at 65°C followed by treatment

with 100 μ l of 5 M NaCl and 100 μ l of CTAB/NaCl (4.1 gr NaCl in 80 ml distilled water with 10 gr CTAB) solution. Subsequently, 750 μ l of chloroform-isoamyl alcohol (24:1) was added and mixed gently and centrifuged at 6,500 rpm for 15 min. The supernatant was transferred to a new microfuge tube and ice-cold isopropanol was added at equal volume. The solution was cooled to -20°C for two hours and DNA was precipitated by centrifugation at 6,500 rpm for 15 min. The isopropanol was discarded and sedimented DNA was dissolved in 1X TE buffer. The concentration of extracted DNA was determined by NanoDrop (Nano Drop, USA) (14).

PCR. A pair of forward (Pf: 5'-ACG GTG GGT ACT AGG TGT GGG TTT C-3') and reverse (Pr: 5'-TCT GCG ATT ACT AGC GAC TCC GAC TTC A3-') primers were used to amplify a 543 bp fragment of 16S rRNA gene specific to Mycobacterium (15 & 16). PCR reactions consisted of 5 μ l of 10x PCR buffer, 4 μ l dNTP mix (each dNTP; 2.5 mM), 1 μ l of each primer (5pm/ μ l), 0.25 μ l Taq polymerase (1.25 U) and approximately 100-150 ng of template DNA. The conditions used for PCR were 10 min at 95°C, followed by 25 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C and final extension at 72°C for 10 min (15 & 16). For 10 isolates a 294-bp region of the hsp65 gene was amplified with primers HSP-1 (Pf: 5'-GCC AAG AAG ACC GAY GAC GT -3') and HSP-2 (Pr: 5'-GGT GAT GAC GCC CTC GTT GC3- ') that have been developed for several mycobacterial genes. PCR was carried out in a final volume of 50 μ l and the thermal profile consisted of an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30s, 62°C for 30s, and 72°C for 1 min and a final period of extension of 6 min at 72°C. The PCR products were loaded into ethidium bromide-stained agarose gels and electrophoresed. Then purification of PCR product was done (15 & 16).

Results: The first growth colonies were observed 10 days after inoculation inclusions (Fig. 2). In microscopy, 30 isolates out of 50 were positive in acid-fast microscopy. The characteristic 543 bp amplicons were observable in PCR amplification of DNA from 30 isolates (Fig. 4). The typical 294- bp amplicon was in PCR amplification of ten isolates (Fig. 6). The result shows that from 50 aquarium fish which were under study, there were 30 acid- fast bacteria and all isolations with mycobacterium



Fig. 1. Autopsy of a mycobacteriosis -suspected fish.



Fig. 2. Mycobacterial cauliflower growth on lowenstein-Jensen slopes.

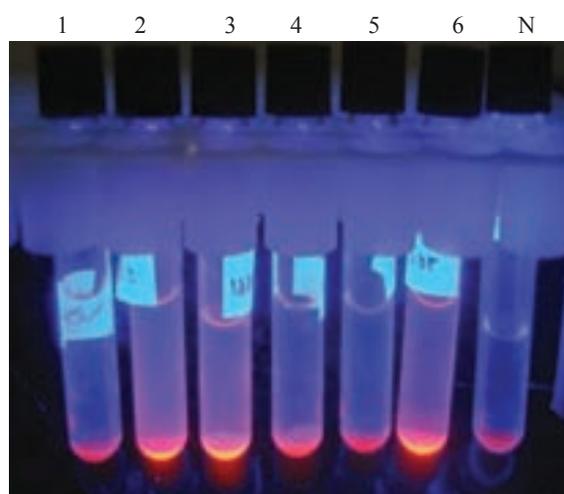


Fig. 3. MGIT Tubes: Tubes 2, 3 and 6 positive, tubes: 1, 4 and 5 negative and N: negative control



Fig. 4. PCR amplification product of the 543-bp specific fragment from 16SrRNA .Lanes 3-16, Mycobacterium species isolates collected from fishes in the study presented here. Lanes M, DNA size marker with 100-bp. Lane N, Negative control. Lane P, positive control.

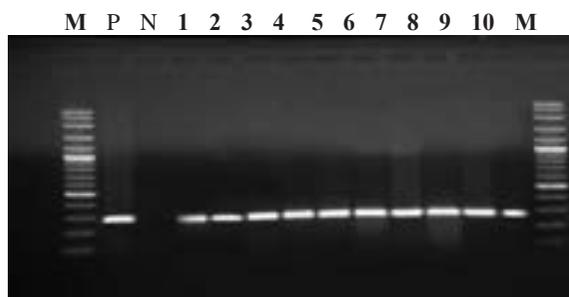


Fig. 5. PCR amplification product of the 294-bp specific fragment from hsp65 .Lanes 1-10, Mycobacterium species isolates collected from the fish in the study presented here. Lanes M, DNA size marker with 100-bp. Lane N, Negative control. Lane P, positive control.

specific primers were positive. The outcomes of the nucleotide sequencing were organized by Chromas, Clustal X and Clustal w programs (Fig. 6) and compared in Gene Bank. Similarity in sequences from 10 isolates and *Mycobacterium fortuitum* from sea creatures was confirmed.

DISCUSSION

Mycobacterium marinum and so other *Mycobacterium* spp. were reported in caviar fishes and fisherman of Caspian Sea in Iran (17). Also some types of mycobacteria in sediments of fish breeding pools and some rapid growing mycobacteria from soil and water in Iran have been reported (18 & 19).

The abundance of Mycobacterium in fish pools is also important due to the general health. While the soil and water have direct contact, so the microorganisms of the soil pollution with Mycobacterium can lead to water pollution. In fish pools specifically where the rim and the floor are muddy, the water pollution of the pool may pollute the fish which have been farming there. Therefore the people who are working

with fish or sediments and the water may become contaminated and many of the fishermen who catch fish with the hands and people who have aquariums at home and housewives who clean contaminated fish are at risk, too (17).

Up to now, only a few occasions of Mycobacterium analysis about fish has been reported in Iran, which was seen as a granuloma on hand of people who deal with aquarium and has been briefed only by clinical witness. There have been some studies in Iran on the presence of *Mycobacterium marinum* and other environmental mycobacteria. The prevalence of *M. marinum* demonstrated to be 7.2% in soil in Mazandaran (18), 1.6% in breeding pool in Gilan (18) and 1.2% from soil in golestan province (17).

In the modern world, more than 70% of pet -raising is belonging to ornamental fish farming. According to the statistics published by FAO, by the year 2004, 188 countries worked in exporting and importing the ornamental fish. Based on this information the main pole in ornamental fish production is the south eastern Asia. The most production rates are in Thailand, China, Malaysia, Indonesia and Singapore. On the other hand, the most amount of ornamental fish importing according to the current statistics is in order belonging to Greece, Hungary, Ireland, and Iran.

Major parts of ornamental fish in Iran are the imported fish. Only some of the ornamental fish are cultivated in Iran. Currently, many live ornamental fish are imported every week via airfreight to Iran from different countries especially from the south East Asia. Sometimes fish which are reproduced inside the country and the imported ornamental fish carrying disease agents are sold to the final consumer/owner, well before any disease would become apparent. To deal this problem, typical tropical fish wholesalers and retailers have no bio-security procedures. The diffusion of the ornamental fish market and consequently the increased international trade of ornamental fish from all over the world have undoubtedly elicited the spread of various and unusual infections internationally (3 & 19). This study emphasizes the fact of existing mycobacterium in Iran's sea creatures and the possibility of transferring to people and causing skin diseases.

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fortuitum	GAGATCGAGCTGGAGGACCCGTACGAGAAGATCGGCGCTGAGCTGGTCAAAGAGGTGCGC 60
Fish1hsp65	-----GCC 3
Fish2hsp65	-----GCC 3
Fish3hsp65	-----GCC 3
Fish4hsp65	-----GCC 3
Fish5hsp65	-----GCC 3
Fish6hsp65	-----GCC 3
Fish7hsp65	-----GCC 3
Fish8hsp65	-----GCC 3
Fish9hsp65	-----GCC 3
Fish10hsp65	-----GCC 3
fortuitum	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 120
Fish1hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish2hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish3hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish4hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish5hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish6hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish7hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish8hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish9hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
Fish10hsp65	AAGAAGACCGATGACGTCGCGGGCGACGGCACCACCACCGCCACCGTTCTGGCACAGGCC 63
fortuitum	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 180
Fish1hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish2hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish3hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish4hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish5hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish6hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish7hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish8hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish9hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
Fish10hsp65	CTGGTTCGTGAAGGTCGCGCAACGTCGCTGCCGGCGCCAACCCGCTCGGCTGAAGCGC 123
fortuitum	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 240
Fish1hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish2hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish3hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish4hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish5hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish6hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish7hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish8hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish9hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
Fish10hsp65	GGCATCGAGAAGGCCGTCGAGAAGGTCACCGAGACGCTGCTGAAGAGCGCCAAGGAGGTG 183
fortuitum	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 300
Fish1hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish2hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish3hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish4hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish5hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish6hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish7hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish8hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish9hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
Fish10hsp65	GAGACCAAGGAGCAGATCGCTGCCACC GCCCGGTATCTCCGCCGGTGACCAGTCCATCGGT 243
fortuitum	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC 360
Fish1hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish2hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish3hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish4hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish5hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish6hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish7hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish8hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish9hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294
Fish10hsp65	GACCTGATCGCCGAGGCCATGGACAAGGTCGGCAACGAGGGCGTCATCACC----- 294

Fig. 6. Alignment of nucleotide sequencing with Chromas, Clustalx and Clustal w programs.

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