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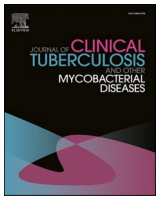

Isolation and identification of non-tuberculous mycobacteria from aquarium fish in Ilam, Iran

Mohammad Yazdanmanesh, Keyvan Tadayon, Darya Bagherian Koshkghazi, Nader Mosavari *

Bovine Tuberculosis Reference Laboratory, Agricultural Research, Education and Extension Organization (AREEO), Razi Vaccine and Serum Research Institute, Karaj, Iran

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ABSTRACT

Non-tuberculous mycobacteria (NTM) are among the most important pathogens in wild, captive, marine, and freshwater fish species. So, it is important to consider fish as the primary source of infection for aquarium fish and humans. The present study analyzed the occurrence of NTM in aquarium fish in Ilam, west of Iran. In total, 50 samples of infected fish were collected from different aquariums. Following initial sample processing, sediment of each sample was inoculated into Lowenstein-Jensen and Herrold egg media. The positive colonies were investigated with, growth rate, pigmentation, colony morphology, niacin accumulation, nitrate reduction, catalase activity, urease activity, and arylsulfatase activity. Also, molecular identification was carried out by sequencing of heat shock protein 65 kD gene (*hsp65*) sequence analysis. According to our results, NTM were isolated from 13 samples (26%), comprising 6 (46.2%) rapid growing, and 7 (53.8%) slow growing mycobacteria. In addition, *Mycobacterium marinum* was the most common NTM isolated in ornamental fish, which is potentially dangerous for both fish and humans. In conclusion, the current study indicates that ornamental fish play a significant role as a source of NTM.

1. Introduction

Non-tuberculous mycobacteria (NTM) are a heterogeneous group of rod-shaped, aerobic, non-motile, and slow-growing bacteria whose cell walls have a high lipid content [1,2]. NTM are environmental opportunistic pathogens of humans and animals and can commonly be present in aquatic and terrestrial environments [3]. Fish mycobacteriosis due to mycobacterial infection was first described in 1897 [4]. This infection is a granulomatous disease that has been reported to occur worldwide in more than 200 species [5,6]. A variety of rapid growing mycobacteria (RGM) and slow growing mycobacteria (SGM) have been isolated from fish, but the most common causes of infections in fish are *Mycobacterium marinum*, *Mycobacterium chelonae*, and *Mycobacterium fortuitum* [4,6]. *M. marinum*,*M. fortuitum*, and *M. chelonae* were initially isolated from saltwater fish (in 1926), neon tetra (in 1953), and Chinook salmon (in 1977), respectively [7–9]. Currently, no effective and definitive treatment eliminates NTM in fish. Also, there is no non-lethal test available to screen fish for this disease [10,11].

Clinical symptoms in fish are nonspecific and include open lesions and ulcerations, lethargy, swollen abdomen, exophthalmia, pile gills, scale loss, red lesions on the lateral line, pigment changes, and strange behavior. At necropsy, may also reveal grey or white nodules in the internal organs, organomegaly in the kidney, liver, and spleen [6,12,13]. The zoonotic nature of the organism, the lack of effective treatment, and the considerable economic losses suffered by the aquaculture industry as a result of this disease highlight the need for rapid detection of NTM and identification at the species level [14,15]. In humans, NTM infections can be chronic and debilitating, often requiring long-term treatment regimens. Chronic pulmonary infection is the most common clinical manifestation caused by NTM [16,17].

Acid-fast bacilli (AFB) stained smears are the first step to the detection of mycobacterial infections [18]. Nevertheless, species differentiation of NTM is essential. Traditional identification, including culture and biochemical analysis, was usually used for the identification of NTM. However, the slow growth of mycobacteria, the complexity and uncertainty of biochemical analysis have made the results time-consuming and confusing [19]. In addition, molecular methods such as hybridization DNA probe assays, 16S rRNA gene multiplex Polymerase Chain Reaction (PCR), or PCR Restriction Enzyme Analysis (PRA) for the identification of NTM, might fail to distinguish closely

* Corresponding author at: Bovine Tuberculosis Reference Laboratory, Agricultural Research, Education and Extension Organization (AREEO), Razi Vaccine and Serum Research Institute, Karaj, Iran.
E-mail address: nmosavari@gmail.com (N. Mosavari).

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related species [20]. As a result, gene sequencing is regarded as the gold standard for identification of NTM. Heat shock protein 65 kD gene (*Hsp65*) is present in all NTM and is frequently used for the identification of NTM to species level due to its interspecies variability compared to some other conserved genes like 16S rRNA and *rpoB* [21]. This study aims to identify NTM isolated from aquarium fish in Ilam, west of Iran.

2. Material and methods

2.1. Sample collection

In total, 50 samples of infected fish were collected from different aquariums during May 2022- January 2023. These included *Trichogaster chuna*, *Channa striata*, *Myrichthys maculosus*, *Puntius tetrazona*, *Helostoma temminckii*, *Pterophyllum scalare*, *Poecilia sphenops*, *Carassius auratus*, and *Labidochromis caeruleus*. The fish were submitted to the laboratory of the Tuberculosis Department in the Razi Vaccine and Serum Research Institute, Karaj, Iran.

2.2. Sample preparation and culture

The samples were homogenized and decontaminated with NaOH and HCL as previously described [22]. In brief, dermal and internal tissue samples were homogenized in a mortar, in small fish (<5 gm in weight), the whole fish was used. Before decontamination, smears of homogenized samples were stained with Ziehl-Neelsen (ZN) and Fluorochrome to identify AFB (Fig. 1). The homogenized tissues were decontaminated for 30 min with NaOH. Then neutralization was done by HCL with 2.5 % phenolphthalein as an indicator. The samples were centrifuged at 3,000 g for 30 min. In the following, 100 μl of sediments were inoculated on the following media: Lowenstein-Jensen with glycerine (LJ), Lowenstein-Jensen with pyruvate (LP) (Razi Vaccine and Serum Research Institute, Karaj, Iran), Herrold egg without and with mycobactin (Razi Vaccine and Serum Research Institute, Karaj, Iran) (Fig. 2). The cultures were incubated at 25 °C and 37 °C for 2 months, as well as the growth ability, colony morphology, and pigment production were monitored twice per week. For acid-fast positive colonies, niacin accumulation, nitrate reduction, catalase activity, urease activity, and arylsulfatase activity were carried out according to the standard instructional [23].

2.3. Molecular identification

2.3.1. DNA extraction

Genomic DNA was extracted from colonies based on Van Soolingen's method using lysozyme, TE buffer, and proteinase K as previously described [24]. The quality of genomic DNA was quantified by Nanodrop spectrophotometry (ND-1000 WOC, Thermo Fisher Scientific, United States).

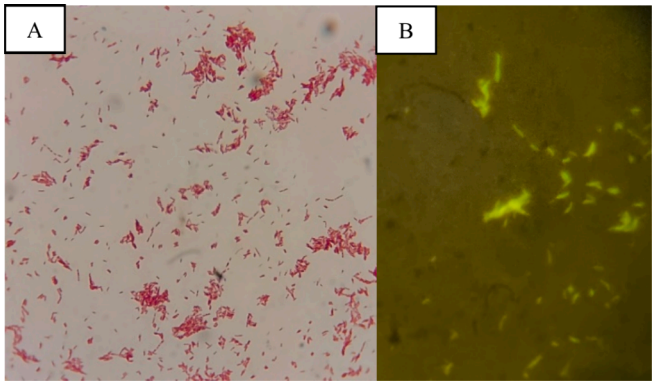

Fig. 1. (A) Ziehl-Neelsen and (B) Fluorochrome staining of hemogenized samples showing acid fast bacilli bacteria.

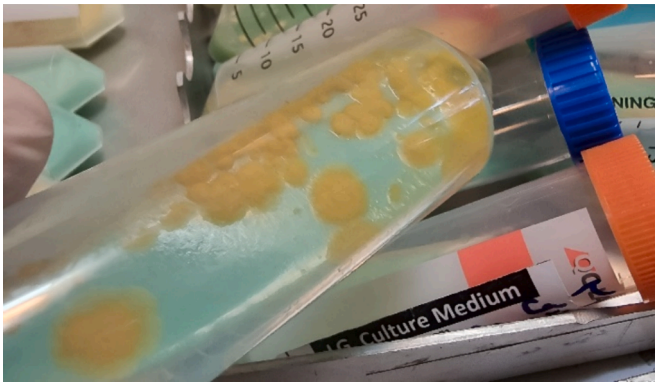

Fig. 2. Colonies of NTM on Lowenstein–Jensen medium.

2.3.2. PCR amplification

A 441 bp fragment of the *hsp65* gene was amplified by PCR using the primers Tb11 (5′-ACCAACGATGGTGTGTCCAT-3′) and Tb12 (5́-TCTGCGATTACTAGCGACTCCGACTTCA-3́) as previously designed [25].

For each sample, PCR was performed in 50 μL volume containing 25 μL PCR Master Mix (SinaClon, Tehran, Iran), 5 μL of the extracted DNA sample (20 ng/μL), 3 μL Dimethyl sulfoxide, 2.5 μL of each primer (10 pm/μL), and finally 12 μL distilled water suitable for molecular tests. Genomic DNA of *M. marinum* (NCIMB 1297) and double distilled water were used as positive and negative controls, respectively. Cycling conditions for the PCR reaction were 94℃ for 5 min, followed by 35 cycles of 94℃ for 1 min, 60 °C for 1 min, 72 °C for 1 min, and finalized with 72 °C for 10 min [25]. The amplification was performed in a thermocycler (Eppendorf PRO S 6325 Thermal, Germany).

2.3.3. Sequencing of the hsp65 PCR product

Double-stranded PCR amplicons from the 441 bp fragment of the *hsp65* products were purified by using a GenUP™ PCR Cleanup Kit and quantified by Nanodrop spectrophotometry spectrophotometry (ND-1000 WOC, Thermo Fisher Scientific, United States), before being sequenced.

2.3.4. Sequence data and phylogenetic analysis

The sequences of *hsp65* gene for each isolate were aligned separately and compared with published nucleotide sequences available in the National Center for Biotechnology Information by BLASTn analysis [14]. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method and Kimura two-parameter (K2P) distance correction model with 1000 bootstrap replications supported by the MEGA 7 software (Fig. 3) [26].

3. Results

3.1. Phenotypic tests

A total of 50 ornamental fish were investigated throughout this study, and 13 NTM were detected by phenotypic tests. According to our results, from 13 NTM, 6 (46.2 %) species of RGM, and 7 (53.8 %) species of SGM were detected. Also, 6 NTM (46.2 %) were identified as *M. fortuitum complex*, and the remaining species could not be identified using this method (Table 1).

3.2. Molecular identification

Positive isolates were identified using sequencing of a 441 bp fragment of the *hsp65* gene, and all of them were easily discriminated from

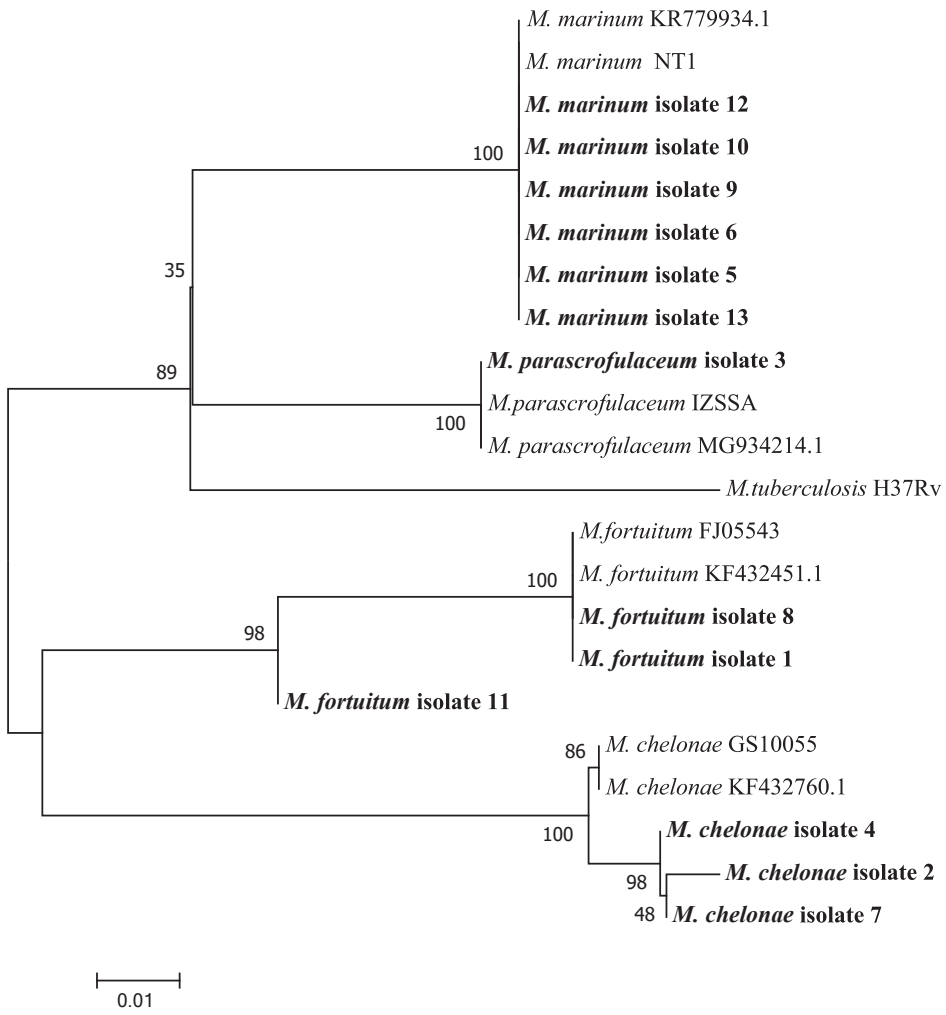

Fig. 3. Phylogenetic tree of the hsp65 gene of isolates NTM from aquarium fish prepared by using the NJ analyses and K2P distance correction model. The support of each branch, as determined from 1000 bootstrap samples, is indicated by percentages at each node. Bar 0.01 substitutions per nucleotide position.

each other. Based on sequencing, *M. marinum* was the most frequently identified species (n = 6, 46.2 %), followed by *M. fortuitum* (n = 3, 23 %), *M. chelonae* (n = 3, 23 %), and *M. parascrofulaceum*, (n = 1, 7/7%) (Table 1). Following the sequencing, all RGM and SGM had the best similarity to their corresponding species in the databanks. The phylogenetic relationships among these thirteen isolates are presented in (Fig. 3).

4. Discussion

In the current study, phenotypic and molecular methods have been used for the detection and identification of NTM. Overall, NTM were detected in 26 % of samples, of which 6 (46.2 %) were RGM and 7 (53.8 %) were SGM. Compared to other investigations, our recovery percentage is lower [4,6,13,27,28]. The results we obtained indicated that species identification by molecular methods (sequencing of the *hsp65*) was more reliable and precise than the phenotypic methods, as previously reported by other investigators [29–31]. Sequence-based methods have been suggested to get high precision and resolution in the identification of NTM compared to other assays [32].

All 13 NTM isolated in the current study were previously isolated from ornamental fish. In this study, the most identified species were *M. marinum* and *M. fortuitum.* However, the occurrence of isolated NTM has been different in other investigations. In India, Shukla et al., examined 60 aquarium fish from six aquarium shops in different cities. According to their report, NTM were detected in 15 (25 %) samples, with *M. gordonae* and *M. abscessus* being the most common NTM isolated [19]. In studies conducted by Hongslo et al., in Sweden, from 120 aquarium fish, NTM were identified in 28 (23 %) samples, and *M. marinum*, *M. chelonae*, *and M. gordonae* were the most frequent species [33]. In other studies in Slovenia by Pate et al., 35 aquarium fish were investigated, and 23 isolates (79.3 %) of NTM were detected, the most prevalent species were *M. fortuitum* and *M. gordonae* [13]. In India, Saha et al., from 161 fish, 132 (81/98 %) AFB were detected [34]. In other reports in the Czech Republic by Beran et al., from 65 samples, NTM were found in 49 (75.4 %) samples, with *M. fortuitum and M. flavescens* being the most commonly isolated [10]. A possible explanation for the variation in NTM prevalence among various regions is the endemic presence of specific NTM in water supply systems. For instance, in the Czech Republic, *M. kansasii* has been identified as an endemic species in water [10].

NTM are opportunistic environmental pathogens that are known to be pathogenic in humans and animals, especially in people with Acquired Immunodeficiency Syndromes [3,35]. Various reports from Iran have shown that *M. fortuitum* was found to be the dominant species in both water and clinical samples [29,36–40]. *M. marinum*,*M. chelonae*, and *M.fortuitum* species isolated from fish in the present study are known as the most common species identified in water samples [5,11,41]. Additionally, all species isolated in this study, notably *M. marinum*, are considered to be pathogenic in both fish and humans [42,43]. *M. parascrofulaceum* isolated from one fish in the current study is a known fish pathogen also related to pulmonary tuberculosis in humans

Table 1
Results of NTM identification by phenotypic and molecular test.

Number of isolates	Fish species	Identification by	
		Phenotypic tests	*hsp65* gene sequence
1	Pterophyllum scalare	*M. fortuitum complex*	*M. fortuitum*
2	Poecilia sphenops	*M. fortuitum complex*	*M. chelonae*
3	Poecilia sphenops	*Mycobacterium* spp.	*M. parascrofulaceum*
4	Pterophyllum scalare	*M. fortuitum complex*	*M. chelonae*
5	Poecilia sphenops	*M. marinum* or *M. simiae*	*M. marinum*
6	Pterophyllum scalare	*M. marinum* or *M. simiae*	*M. marinum*
7	Pterophyllum scalare	*M. fortuitum complex*	*M. chelonae*
8	Pterophyllum scalare	*M. fortuitum complex*	*M. fortuitum*
9	Helostoma temminckii	*M. marinum* or *M. simiae*	*M. marinum*
10	Carassius auratus	*M. marinum* or *M. simiae*	*M. marinum*
11	Poecilia sphenops	*M. fortuitum complex*	*M. fortuitum*
12	Poecilia sphenops	*M. marinum* or *M. simiae*	*M. marinum*
13	Labidochromis caeruleus	*M. marinum* or *M. simiae*	*M. marinum*

[44,45].

In conclusion, the findings of the present study suggest that aquarium fish are a source of NTM, which is known to cause disease in fish and can also infect humans. Further studies of NTM and modes of transmission using various molecular methods are needed because these methods can lead to a much better understanding of the global phylogenetic diversity of the NTM and increase the specificity, sensitivity, and accuracy of diagnosis.

Author contributions

N.M and K.T contributed to the study conception and design. Data collection and analysis were performed by M.Y.M. The first draft of the manuscript was written by M.Y.M and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical approval

Ethical approval was obtained from the Razi Vaccine and Serum Research Institute ethical committee, Karaj, Iran (IR.RVSRI.REC.1402.001).

CRediT authorship contribution statement

Mohammad Yazdanmanesh: Writing – original draft, Investigation. **Keyvan Tadayon:** Investigation, Formal analysis. **Darya Bagherian Koshkghazi:** Investigation. **Nader Mosavari:** Writing – original draft, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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