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Research Article

Isolation and molecular identification of *Mycobacterium* spp from aquarium fish in Urmia city, northwest of Iran

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ABSTRACT

Fish mycobacteriosis is a zoonotic and chronic progressive disease that caused by different species of mycobacteria. The aims of this study were isolation and molecular identification of *Mycobacterium* spp from aquarium fish in Urmia city, northwest of Iran. 30 apparently healthy ornamental fishes from different species were collected from five aquarium fish centers and were considered for the isolation and identification of *Mycobacterium* spp by bacterial culture, biochemical tests and PCR assay. 4 (13.3%) samples contained mycobacteria where biochemical tests revealed that 2 isolates were *Mycobacterium marinum* and 2 isolates were identified as *Mycobacterium fortuitum*. All of four isolates were molecularly confirmed by PCR test. The results demonstrated the presence of *Mycobacterium* spp zoonotic bacterial pathogens in healthy *Carassius auratus*, *Puntius conchonius*, *Poecilia latipinna* and *Astronatus ocellatus* fish, and underlined the infection risk to humans of not only exposure to infected fish, but also when they manipulate clinically asymptomatic fish.

1. Introduction

Fish mycobacteriosis is a chronic progressive disease caused by different species of mycobacteria. Some of Mycobacterium species that commonly cause mycobacteriosis in ornamental fish include *Mycobacterium marinum*, *Mycobacterium cheloni* and *Mycobacterium fortuitum*. Furthermore, several other Mycobacteria species have been identified in relation with the mentioned disease (Pate et al., 2005). In addition to causing death in fish, Mycobacterium species can be transmitted to human beings under certain conditions and result in skin infections called fish tank granulomas and swimming pool granulomas (Pourahmad et al., 2009). Non-tuberculous mycobacteria such as *Mycobacterium marinum* can be transmitted from contaminated ornamental fish or related environments (e.g. contaminated water). This could happen while cleaning or handling fish through cuts or scratches on the skin, and lead to infection of humans (Beran et al., 2006; LeBlanc et al., 2012). *Mycobacterium cheloni* and *Mycobacterium fortuitum* are invasive species that grow rapidly and are widely distributed in the environment (soil and water); these are considered as common causes of nosocomial infections, and usually result in superficial

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lesions and possibly lung disease and primary lymphadenopathy (Kent Michael et al., 2016; Mainous and Smith, 2005; Watral and Kent Michael, 2007). This disease in aquarium fish is usually diagnosed by histopathology, bacterial culture, biochemical tests and molecular methods. Of course, the initial diagnosis of fish mycobacteriosis is based on clinical and postmortem examination and finding the granulomas in skin and visceral tissues. However, sometimes the fish have no clinical signs and no granuloma masses are found (Gauthier and Rhodes, 2009). Aquatic animals, including aquarium fish, can act as a source of exposure to human-transmitted pathogens. There are not many reports of outbreaks of zoonotic infections acquired from aquarium fish, and often sporadic cases have been reported. Due to the risk of mycobacterium infections in human as well as economic losses resulting from adverse effects of mycobacterium fish such as reduced feed efficiency, reduced growth and increased mortality, it is crucial to identify mycobacterium in ornamental fish (Zanoni et al., 2008). The present study attempted to isolate and recognize the molecular identity of *Mycobacterium* spp isolates collected from aquarium fish in Urmia city, northwest of Iran.

2. Materials and Methods

2.1. Sample collection

In this descriptive cross-sectional study, thirty apparently healthy ornamental fishes from different species were collected from five aquarium fish centers in Urmia city, northwest of Iran, during nine months (from March to December in 2021) and were subjected for the isolation and identification of *Mycobacterium* spp. 10 species were examined, including: Copper Oscar (*Astronotus ocellatus*), Koi (*Cyprinus rubrofuscus*), Molly black (*Poecilia sphenops*), Flower horn (*Paraneetroplus synspilus*), Dwarf parrot (*Pygmy parrot*), Severum (*Heros severus*), Electricity cichlid (*Labidochromis caeruleus*), goldfish (*Carassius auratus*), Rosy barb (*Puntius conchonius*) and Sailfin molly (*Poecilia latipinna*).

2.2. Isolation and identification of Mycobacterium spp

Fishes were euthanized using an overdose of ethyl 3-aminobenzoate methane sulfonate (Sigma Aldrich, USA). Skin, gut content, and visceral organs (spleen, liver, and kidney) were taken from each fish. If sampling the gut content was not possible due to their small size, entire guts were subjected to the cultivation protocol. Before decontamination, smears of homogenized biological material were prepared, stained with Ziehl-Neelsen (ZN), and observed under a 100x (oil immersion) objective lens for the detection of Acid-fast bacilli (AFB). A negative report was not given until at least 100 fields had been examined. For mycobacterial culture, the samples were mixed with an equal volume of a 5% oxalic acid solution and incubated for 15 min. Afterward, the samples were centrifuged at 3,000 g for 15 min. The pellets were washed twice in sterile phosphate buffered saline and inoculated onto one egg Lowenstein-Jensen (LJ) media at 25°C and 37°C. The slants were checked daily for 2 months. Visible colonies were examined according to their morphology and confirmed by microscopic examination after ZN staining of smears prepared from the colonies (Puk and Guz, 2020). Detection of isolated *Mycobacterium* spp was performed using phenotypic characteristics (growth rate, growth at different temperatures, growth in MacConkey agar medium, colony morphology, pigment production test) and biochemical tests (niacin and urease production, heat resistant catalase test (at 68 ℃), hydrolysis of Tween 80, 3-day aryl sulfatase test, 5% sodium chloride tolerance test and nitrate reduction test) (Moghim et al., 2012).

2.3. DNA extraction

The cetyltrimethylammonium bromide (CTAB) was used for DNA extraction in *Mycobacterium* spp isolates. One ml of bacterial culture was well dissolved in 400 µl of Tris-EDTA buffer. Afterward, 30 µl of lysozyme (50 mg/ml) were added to it and they were incubated for 2 h at 37 ℃. 10 µl of proteinase k and 70 µl of 10% SDS (Sodium dodecyl sulfate) were added to it and they were mixed well and then, placed in water bath at 65 ℃ for 15 min. In the next step, 100 µl of NaCl (5 M) was added and also 100 µl of CTAB, which had already been heated to 65 ℃, was added to it and then, placed in water bath at 65 ℃ for 10 min. Then, 700 µl

of phenol chloroform (with equal volume) was added to the microtube and centrifuged at 13,000 g for 15 min. After the supernatant was transferred to the new microtube, 500 µl of cold isopropanol was added to the microtube and kept in -20 °C for 30 min. It was then centrifuged at 13,000 g for 20 min and isopropanol was poured off. Finally, 1 ml of 70% cold ethanol was added and centrifuged at 13,000 g for 20 min. Ethanol was carefully poured off and the excess alcohol evaporated (Rezaeyan et al., 2016).

2.4. PCR test to confirm the molecular diagnosis of Mycobacterium spp

The polymerase chain reaction (PCR) method was done in 25 µl, including 11µl of Master mix PCR, 1 µl of each specific primers (25 nanomole) (16s rRNA Forward: 5′- ACG GTG GGT ACT AGG TGT GGG TTT C- 3′; Reverse: 5′- TCT GCG ATT ACT AGC GAC TCC GAC TTC A- 3′) (Ghaderi et al., 2020), 1 µl (50 ng) of DNA template and 11 µl of double distilled water. Time table and thermal schedule for the intended gene were as follows: primary denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, extension at 72 °C for 1 min and a final extension cycle at 72 °C for 10 min. The amplified product was run on 1.5% agarose gel and stained with ethidium bromide (0.5 mg/ml) in a dark room.

3. Results

According to the bacterial culture, out of 30 tested ornamental fishes, 4 (13.3%) samples contained mycobacteria (from 4 different aquarium fish centers). The biochemical tests revealed that 2 isolates were *Mycobacterium marinum* and 2 isolates were identified as *Mycobacterium fortuitum*. *Carassius auratus* and *Puntius conchonius* were each infected with *Mycobacterium marinum*. *Poecilia latipinna* and *Astronatus ocellatus* were each infected with *Mycobacterium fortuitum*. All of four isolates that had been identified as *Mycobacterium* spp were molecularly confirmed by PCR test (Figure 1).

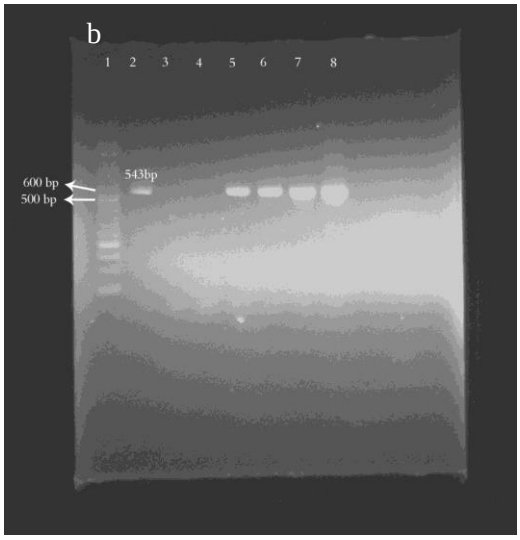

Figure 1. Lane 1 is marker (50 bp). Lane 2 indicates BCG (1173 P73) as positive control that bands within 543 bp and is associated to *Mycobacterium* spp. Lane 3 is negative control (Double distilled water). Lanes 5-8 are positive samples.

4. Discussion

Mycobacteria have a widespread distribution, and these organisms have also widely lived in aquatic environments. Mycobacteriosis is one of the most common infections of ornamental fish; this has been reported in more than 150 species (Kankya et al., 2011; Mainous and Smith, 2005). When fish are infected with Mycobacteria, they usually do not behave differently from non-infected fish. They only become suspected when their motility is impaired. The skin color of the fish fades, and other clinical symptoms such as skin inflammation, skin lesions and exophthalmos occur (Salawudeen et al., 2017; Shukla et al.,

2013). In current study, out of 30 ornamental fishes tested (among 10 different species), 4 (13.3%) were infected with *Mycobacterium* spp. *Carassius auratus* and *Puntius conchonius* were infected with *Mycobacterium marinum*. Also, *Poecilia latipinna* and *Astronatus ocellatus* were infected with *Mycobacterium fortuitum*. These findings agree with the results of Seyfahmadi et al (2018) (Seyfahmadi et al., 2018). Akbari et al (2014) reported that out of 50 ornamental fish tested in Alborz province (north of Iran), 20% were infected with *Mycobacterium fortuitum* (Akbari et al., 2014). Sirimalaisuwan et al (2017) found that 8.42% of Siamese fighting fish (*Betta splendens*) from ornamental fish shops in Chiang Mai Province, Thailand were infected with *Mycobacterium* spp and 5.5% isolates were identified as *Mycobacterium marinum* (Sirimalaisuwan et al., 2017). *M. marinum*, *M. fortuitum* and *M. piscium* are the most common species of mycobacterium in fish. Numerous species of tropical pet fish have been reported with mycobacterial infections. These include dwarf cichlid (*Apistogramma cacatuoides*), goldfish (*Carassius auratus*), angelfish (*Pterophyllum* sp.), guppy (*Poecilia reticulata*) and zebrafish (*Danio rerio*) (Kušar et al., 2017; Slany et al., 2014). Conventional bacterial culture methods used to diagnose *Mycobacterium* spp. are slow and rely only on phenotypic characteristics; this can be hard to differentiate from other mycobacterial species such as the non-mycobacterial bacteria. *Nocardia* spp. and *Actinomyces* spp. also show acid-fast property (Ahsan et al., 2015). However, in our study, PCR results showed that all of the acid-fast bacilli grown on culture media were *Mycobacterium* spp. DNA amplification from the highly conserved region of 16S rRNA is very useful for identifying *Mycobacterium* spp. Exposure to contaminated water and superficial cuts or abrasions of the skin are two major risk factors of *M. marinum* and *M. fortuitum* infections in non-immunocompromised patients (Slany et al., 2013; Johnson and Stout, 2015). Ornamental fish owners and others with high risk of *M. marinum* and *M. fortuitum* infections should be educated about the zoonotic risk associated with handling aquarium fish and the aquarium environment.

Conclusion

In this study, *M. marinum* and *M. fortuitum* which are well-known pathogens in fish and human, were isolated from ornamental fish. Due to the zoonotic disease and the difficulty of treatment and the need for long-term treatment, people who deal with aquarium fish, especially those with immunodeficiency, should pay more attention to keeping aquarium fish to prevent or reduce disease transmission to human beings.

Conflict of interest

We declare that we have no conflict of interest.

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