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Phenotypic and Molecular Characterization of *Mycobacterium* species from Fresh Water Aquarium Fish and Water Samples

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Mycobacteriosis is a serious chronic disease that affects a wide range of marine and fresh water ornamental fish. The present study analyzed the incidence of mycobacteria in 60 apparently healthy fresh water aquarium fish belonging to eight different species as well as 10 pond water samples from Uttar Pradesh, India. In total, thirteen mycobacterial strains of nine species were recovered, out of which, eleven isolates belonging to seven species were obtained from fish and two isolates of different species from pond water. The proportion of mycobacterial incidence was reported 18.33% in apparently healthy fresh water aquarium fish and 20% in pond water. Isolates were identified on the basis of growth characteristics, phenotypic characters and sequence analysis of the *16S rRNA* gene as: three isolates of *M. abscessus* two isolates of *M. fortuitum* two isolates of *M. conceptionense* and one isolate of *M. parascrofulaceum, M. gordonae, M. bolletii, M. paraffinicum, M. aubagnense, M. arupense* each. Mycobacterial incidence in clinically healthy freshwater aquarium fish and natural water sources is awful and the study is relevant because of the mycobacterial diversity related to aquarium fish and its zoonotic importance.

Key words: Mycobacteria; Aquarium fish; Pond water; Biochemical; 16S-*rRNA*; Zoonosis.

Mycobacteria are pleomorphic, gram-positive, acid-fast, aerobic, non-motile bacteria with surprisingly diverse phenotypes related to growth rate, colony appearance, environmental distribution, and pathogenic potential for eukaryotic hosts[1]. To date, the genus *Mycobacterium* comprises over 150 species (*http://www.bacterio.cict.fr/m/mycobacterium*). The traditional method of identification of mycobacteria from clinical and environmental sources includes isolation using enrichment and selective media and subsequent screening and confirmation by the phenotypic characteristics of biochemical testing, pigment production and growth characteristics, colony morphology[2]. With almost 150 currently established species, a number that continues to rise, biochemical algorithms become too complex, which results in an inherent bias towards the identification of more familiar species of mycobacteria. Molecular methods such as PCR offer a significant alternative for rapid selection and identification of these bacteria. In recent years a well-established sequencing analysis using *16S rRNA* gene has greatly contributed to an accurate identification and classification of mycobacteria[3]. Several new species have been identified by *16S rRNA* sequencing which could not have been identified by conventional methods[4].

Mycobacteriosis has been reported to affect a wide range of freshwater and marine fish species suggesting ubiquitous distribution[5]. Mycobacterial diseases rank among the most prevalent chronic diseases in tropical and sub-

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tropical freshwater aquarium fish[5,6]. *Mycobacterium* sp. infect over 160 species of salt water and fresh water fishes[7]. Factors that may predispose fish to mycobacterial infection include dietary or environmental contamination, stress associated with overcrowding, suboptimal nutrition, and poor water quality[8].

In general, mycobacteriosis in fish has been described as a systematic, chronic, progressive disease with clinical signs that include skin discoloration, loss of appetite, lethargy, abnormal swimming behavior, isolation, cutaneous ulceration or erosions, ascites, reduced growth and exophthalmia[9]. The economically significant consequences of these infections include mortality, morbidity and effects of subclinical infection such as decreased feed efficiency, decreased growth rates, and decreased marketability[10]. Granulomas enclosing acid-fast rods (AFR) after Ziehl-Neelsen (ZN) staining within various host tissues are a typical characteristic of this infection[11]. Various species of potentially pathogenic mycobacteria (PPM) or environmental saprophytic mycobacteria (ESM) have been detected mostly in fresh water aquarium fish. These include: *M. abscessus*, *M. aurum*, members of *M. avium complex*, *M. celatum, M. chelonae, M. diernhoferi, M. flavescens, M. fortuitum, M. gordonae, M. intra-cellulare, M. kansasii, M. marinum, M. montefiorese, M. nonchromogenicum, M. parafortuitum, M. peregrinum, M. poriferae, M. scrofulaceum, M. simiae, M. terrae* and *M. triviale*[12,13,14].

Mycobacterial infections in fish also present a risk factor for the human populations. People become infected while working with the contents of aquaria with infected fish[11]. This granulomatous disease is also known as fish handler's disease or fish tank-granuloma as it occurs mainly in aquarists[14,15]. There are reports of increasing number of human cases, not only in professional fish handlers, but also in non-professionals who can develop various forms of disease[16]. Among these, cutaneous infections, lymphadenopathy, osteomyelitis and lung disease are the most important[17].

The purpose of the present study was to analyze the incidence and distribution of mycobacterial species in apparently healthy freshwater aquarium fish and aquarium environment.

MATERIALS AND METHODS

Fish and Bacterial sources

Freshwater aquarium fish (n=60) with no observable signs of disease were randomly collected from aquarium shops. Samples consisted of 6 specimens each of *Carassius auratus, Helostoma temminckii, Colisa lolia, Danio rerio, Balantiocheilus melanopterus, Epalzeorhynchos frenatus, Trichogaster chuna* and 18 specimens of *C. auratus auratus*. In addition, 10 environmental samples (Pond water) were collected from different cities in India. All samples were processed for isolation of mycobacteria.

Tissue homogenization and decontamination

Fish were euthanized with an overdose of MS-222 (tricaine methane sulphonate). In case of small fish (<5 gm in weight), whole fish were used, whereas, in larger fish, dermal and internal tissue samples were pooled and homogenized in sterile NSS (normal saline solution) using a tissue grinder. Approximately 2 g of tissue samples in 2ml of NSS were used to prepare homogenates. The tissue samples were decontaminated as per Shitaye[18] whereas, the water samples were decontaminated following Falkinham[19].

Bacterial isolation

Decontaminated homogenates (0.05 ml) were inoculated onto Lowenstein-Jensen (LJ) slants with Gruft's supplement (Hi-media, India). Inoculated slants were incubated at 30°C and examined for 2 months. All morphologically distinct colonies were streaked to Middlebrook 7H11 agar (MDA, Hi-media, India) plates supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC) and 0.5% (v/v) glycerol to obtain pure cultures.

Identification of the isolated Bacteria

Buff, yellow or orange colonies grown within 3 to 60 days were subjected to acid-fast (Ziehl-Neelsen) staining and examined microscopically. Identification of the isolated acid fast bacilli was carried out depending on morphological characters, rate of growth, pigmentation, and growth at different temperature (25°C, 30°C and 37°C)[20].

Purified isolates were characterized using the following biochemical characteristics: growth on LJ with 5% NaCl, pigment production, nitrate reduction, catalase thermo resistance, tween-80

hydrolysis, tellurite reduction, arylsulphatase activity, production of pyrazinamidase and resistance to TCH (Thiophene-2-carboxylic acid hydrazide). All tests were conducted at 30^{0}C and diagnostic keys[20,21] were used to assign isolates to an existing species, when applicable.

Identification by *16S rRNA* gene amplification

DNA preparation

Mycobacterial cells grown in Middlebrook 7H9 broth (MDB) containing ADC enrichment and 0.05% glycerol were pelleted at 5000g for 5 min and used for DNA isolation. Purified DNA was extracted from mycobacterial isolates by CTAB method using lysozyme and proteinase K[22]. DNA quality was assessed by electrophoresis in 0.8% agarose with ethidium bromide staining.

PCR amplification of *16S rRNA* gene

A 924 base pair fragment from the *16S rRNA* gene was amplified using the T39 (5'-GCGAACGGGTGAGTAACACG-3') and T13 (5'-TGCACACAGGCCACAAGGGA-3') primers[23]. Each 50 µl PCR reaction mixture contained a 25µl PCR Master Mix 2X (Thermo Scientific, USA), ultra-pure water (Millique), 1 µM of each primer and 1 µl of the extracted bacterial DNA. Amplification reactions were performed on a Thermal cycler (Bio-Rad Laboratory, USA) and amplification cycle consisted of 5 min denaturation at 95°C followed by 30 cycles of denaturation at 94°C for 1 min; annealing at 50°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. PCR amplification was confirmed by electrophoresis using 2µl of PCR product on 1% agarose gel stained with ethidium bromide and visualization under UV light (Fig.1).

DNA sequencing and phylogenetic analysis

PCR products were sequenced at the DNA sequencing biological services unit SciGenom Kochi, India. Gene-specific primers (T39 and TI3) yielded the complete sequences of both DNA strands and confirmed reading accuracy. Both forward and reverse sequences of all the isolates used in this study were assembled, edited and compared with those of several closely related isolates available in the National Center for Biotechnology Information's GenBank database (*http://www.ncbi.nlm.nih.gov/genbank/*). Obtained *16S rRNA* gene sequences were downloaded and phylogenetic tree was constructed within a group of related mycobacterial species using the neighbour-joining method and Kimura's two-parameter distance correction model. The tree inferred from the *16S rRNA* gene sequences was rooted using *Nocardia asteroides* KC262094 as the out-group. Statistical confidences in tree branches were generated by performing 500 bootstrap replicates.

RESULTS AND DISCUSSION

During this study, a total of 70 samples were investigated. Using the initial protocol of decontamination and incubation at 30^{0}C for 2 months, a wide variety of *Mycobacterium* spp. were recovered. *Mycobacterium* spp. were isolated from 11 of 60 (18.33%) fish examined, representing all eight species of fish sampled. Two additional *Mycobacterium* spp. were isolated from two different pond water samples. These 13 isolates were selected for biochemical identification (Table 1). Then all the 13 isolates were subjected to molecular identification by amplification and sequencing of a 924 bp fragment from a highly conserved region of the mycobacterial *16S rRNA* gene. After sequencing of *16S rRNA*; eleven isolates from fish were confirmed as three isolates of *M. abscessus*, two isolates of *M. fortuitum*, two isolates of *M. conceptionense*, and one isolate of *M. parascrofulaceum, M. gordonae, M. bolletii, M. paraffinicum* each, while the two isolates from pond water samples were identified as *M. aubagnense* and *M. arupense* each. Out of thirteen mycobacterial strains of nine species, five species (*M. abscessus, M. aubagnense, M. fortuitum, M. conceptionense, M. bolletii*) were rapid growers, three (*M. parascrofulaceum, M. gordonae, M. paraffinicum*) were slow growers whereas one (*M. arupense*) grew rapidly at 30°C and slowly at 37°C. The proportion of mycobacterial incidence in apparently healthy fresh water aquarium fish was reported 18.33% and 20% in pond water.

16S rRNA phylogenetic analysis was performed to characterize the phylogenetic relationships of these mycobacteria in more detail. The sequences of *16S rRNA* of isolates of *Mycobacterium* species (MF1, MF2, MF3, MF4, MF5, MF6, MF7, MF8, MF9, MF10, MF11, MF12, MF13) had best matches with 99% to 100% similarity with their corresponding species in the databanks.

Table 1. Phenotypic characterization of isolates

Species hydrolysis	**source** Arylsulphatase activity	Growth at temperatures Catalase 25°C	30°C	Urease 37 °C	Growth Iron rate	Growth Update on LJ with 5% NaCl	Pig- ment ation	Ni Pyrazinamidase tr ate	Red uc tion	Tellurite reduction Resistance to TCH 3days		14days		Tween-80		
M. abscessus (MF1)	*Carassius auratus*	+	+	+	RG	+	N	-	+	+	+	+	+	+	-	+	+
M. aubagnense (MF2)	Water Sample	+	+	+	RG	-	N	-	+	+	+	+	-	-	-	-	+
M. parascrofulaceum (MF3)	*Colisa lolia*	+	+	+	SG	-	S	-	-	-	-	-	+	+	-	+	+
M. arupense (MF4)	Water sample	+	+	+	MG	-	N	-	-	+	-	+	+	-	-	-	+
M. fortuitum (MF5)	*Helostoma temminckii*	+	+	+	RG	+	N	+	+	+	+	+	+	-	+	+	+
M. fortuitum (MF6)	*Danio rerio*	+	+	+	RG	+	N	+	+	+	+	+	+	-	+	+	+
M. conceptionense (MF7)	*Helostoma temminckii*	+	+	+	RG	+	N	+	+	-	+	+	-	+	+	-	+
M. gordonae (MF8)	*Carassius auratus auratus*	+	+	+	SG	-	S	-	-	+	-	+	+	-	-	+	+
M. abscessus (MF9)	*Carassius auratus auratus*	+	+	+	RG	+	N	-	+	+	+	+	+	+	-	+	+
M. bolletii (MF10)	*Balantiocheilus melanopterus*	+	+	+	RG	-	N	-	+	-	+	+	-	-	-	+	+
M. abscessus (MF11)	*Carassius auratus auratus*	+	+	+	RG	+	N	-	+	+	+	+	+	+	-	+	+
M. paraffinicum (MF12)	*Epalzeorhynchos frenatus*	+	+	+	SG	-	S	-	-	-	-	+	+	-	+	-	+
M. conceptionense (MF13)	*Trichogaster chuna,*	+	+	+	RG	+	N	+	+	-	+	+	-	+	+	-	+

RG: Rapid grower, SG:Slow grower, MG: Moderate grower

The isolates *M. abscessus* (MF1), *M. abscessus* (MF9), *M. abscessus* (MF11) and *M. bolleti* (MF10) matched with *M. abscessus* AY457071 and *M. bolleti* AY859681. Isolates *M. conceptionense* (MF7, MF13) had matched with *M. conceptionense* AY859684 and respectevly isolates *M. fortuitum* (MF5, MF6) matched with *M. fortuitum* AY457066. Isolate *M. arupense* (MF4) was matched with *M. arupense* DQ157760 and *M. aubagnense* (MF2) clearly matched with *M. aubagnense* AY859683. Isolate *M. parascrofulaceum* (MF3) clearly matched with *M. parascrofulaceum* AY337273 while *M. paraffinicum* (MF12) matched with *M. paraffinicum* GQ153270 and *M. gordonae* (MF8) highly matched with *M. gordonae* X52923. The phylogenetic relationship among this isolate are presented in Fig 2.

Mycobacteria were isolated from the skin and internal organs of apparently healthy fish. These results were notable because we did not expect to find mycobacteria in such fish with no clinical sign of mycobacterial infections. The isolation of mycobacteria takes a few days in rapidly growing mycobacterial species and several weeks in slow growing species. Mycobacteria are often overgrown by other microorganisms present in the sample, to overcome this obstacle, it is necessary to decontaminate samples before culture. Sample decontamination is required to

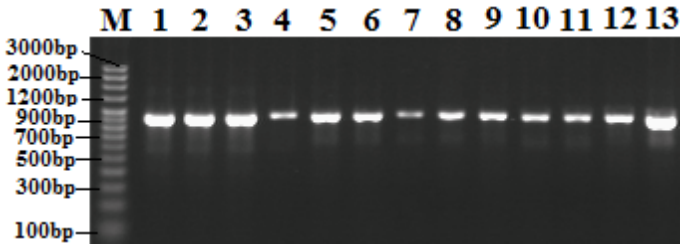

Fig. 1. 16S rRNA Gene amplification of isolated aquatic mycobacteria. PCR products of thirteen samples Lane 1-13. Lane M =100 bp DNA ladder (Fermantas) marker

reduce background overgrowth, to inactivate other bacteria that might be present in the sample, to avoid their faster growth and media nutrients exhaustion[26]. However, identification methods based on culture using solid and liquid media, growth characteristics and biochemical tests are still considered the most reliable and a standard method for more than 100 years but molecular methods are also very useful for identification of mycobacterial species[23-25]. In our study isolate MF10 was identified as *M. abscessus* through biochemical tests but in sequencing results it clearly matched with *M. bolleti* it indicates that molecular characterization is essential for the identification up to species level.

The dawn of molecular methods for identification, particularly sequence-based methods, has exponentially increased the discovery and validation of new mycobacterium species in the last few years. Detection of *Mycobacterium* spp. by polymerase chain reaction in fish is progressing while the reproducibility and accuracy of the PCR method is determined by many factors including the choice of primers, the preparation of the DNA, the amount of target DNA used in each reaction, the presence of natural or induced inhibitors and DNA contamination[27]. This particular DNA encoding *16S rRNA* is a highly conserved part of the bacterial genome comprising sufficient information to allow both phylogenetic analysis and species identification.

In this study we identified the 13 isolates of mycobacteria, covering 9 species, performing sequence similarity searches using the *16S rRNA* gene sequences (924 bp). While we are aware of the possible presence of sequence errors from GenBank data, we have evaluated them in comparison with close relatives and have determined them to be of acceptable quality, i.e., presenting no mismatches in conserved areas and no omissions or insertions out of place.

Although the percentage of samples positive for mycobacteria was not high in our study and it is not certain whether the disease was initial or latent, or whether the fish tissues were merely colonized with mycobacteria present in the aquarium. It can be assumed that the incidence of mycobacterial infections in aquarium fish is

primarily associated with mycobacterial contamination of the aquarium environment. Based on the literature[11,19,26] which describes a high incidence of mycobacteria in water distribution systems and in surface water, their transmission from water sources is projected.

There are few previous studies aimed at investigating the incidence of mycobacteria in a randomly selected sample of clinically healthy fish populations living in their natural environment[17,28]. Most studies involved populations of fish showing clinical signs of mycobacteriosis[29,30,31,32]. Currently, there are approximately 150 recognized mycobacterial species (Euzeby, 2010 *http://www.bacterio.cict.fr/m/mycobacterium.html*)[33] and more than 20 have been isolated from freshwater fish[17]. During this study of freshwater fish, 7 *Mycobacterium* species were isolated which may be considered a wide range of species. *M. fortuitum*, *M. abscessus* and *M. gordonae* isolated from fish in this study are well known pathogens for fish and cause fish tuberculosis[29] and these were also classified as PPM which can cause infections to human. *M. conceptionense* isolated from two samples in present study is one of the most common cause of fish mycobacteriosis[34] and may cause various pulmonary, skin, or soft-tissue infections in humans[35]. *M. parascrofulaceum* is a known pathogen for fish and also related with pulmonary tuberculosis in human[4] similarly, *M. bolleti* was isolated from sputum (three cases) and from a gastric aspirate from patients with chronic pneumonia[35]. Isolation of *M. paraffinicum from patients was reported in a medical* center by most precise method *16S rRNA* gene sequencing[36]. In our study *M. arupense* was isolated from pond water sample and isolation of *M. arupense* from water samples[37,38] as well as from fish[34] was reported previously. *M. aubagnense* isolated from pond water sample may also cause serious infections in humans[34].

The therapy of mycobacteriosis in fish has not yet been satisfactorily developed and is impossible in such cases with no clinical signs. Studies have shown that prevention plays a critical role in controlling mycobacterium infections in

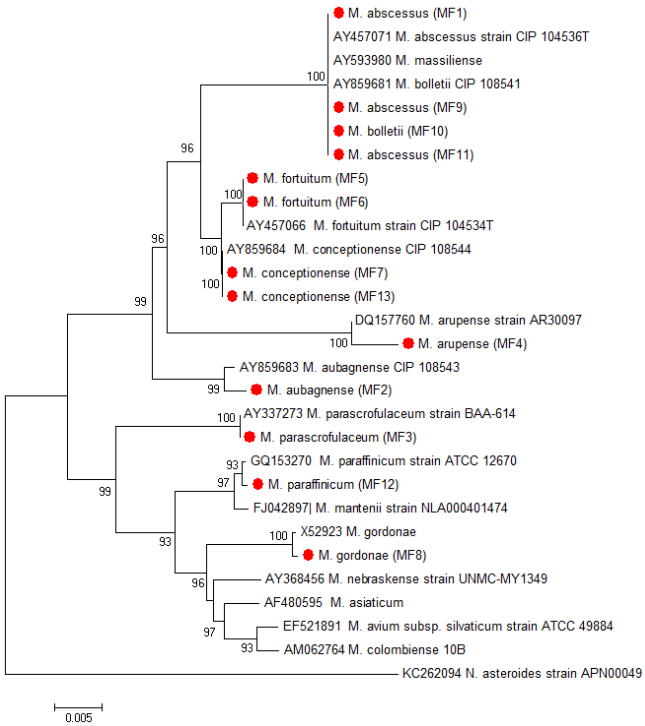

Fig. 2. Phylogenetic tree of the *16S rRNA* gene of isolated mycobacteria prepared by using the neighbour-joining method and Kimura's two-parameter distance correction model. The support of each branch, as determined from 500 bootstrap samples, is indicated by the value at each node (as a percentage). Bootstrap values less than 70% are not shown. *Nocardia asteroides* KC262094 was used as the out-group. The scale bar indicates a 0.5 % difference in nucleotide sequences

fish[19]. This prevention consists primarily of maintaining good water quality and sufficient fish culture sanitation. In commercial aquarium fish breeding facilities, prevention also includes regular veterinary inspection, which is necessary for both imports of fish from abroad as well as transport within India. Moreover, it is necessary to minimize the stress when fish are removed from natural environments and placed in aquaria.

The occurrence of mycobacterial infections in asymptomatic freshwater aquarium fish could be a serious problem in India. In addition to fish tuberculosis, these nontuberculous mycobacteria are capable of causing both localized and disseminated infections in humans, in addition to significant economic losses in aquaculture. It would appear that diagnosing mycobacterial infections based solely on clinical symptoms of disease or the presence of granulomas is inadequate and more complete diagnostic workups, including long-term culture and molecular analysis. All species of mycobacteria isolated in this study are potentially pathogenic to humans. As such, infected aquarium fish represent a risk factor for human populations, especially amongst cohorts in the commercial fish industries and hobby aquarists.

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