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Characterisation of Non-tuberculous Mycobacteria Isolated from Apparently Healthy and Diseased Fresh Water Ornamental Fish in Sri Lanka

D.R. ANURUDDHIKA DISSANAYAKE[1*], VINDYA PERERA[1], S.S. SAMANTHIKA DE. S. JAGODA[1], E.A. ROSHANI EDIRISINGHE[1] and APPUDURAI ARULKANTHAN[1]

[1]Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya, Sri Lanka

Abstract

Eighty nine fish including guppies (n=55), Siamese fighting fish (n=12), Swordtails (n=10), Platies (n=6) and goldfish (n=6) showing emaciation, scoliosis and loss of pigmentation, and 30 apparently healthy guppies collected from pet shops and fish breeding farms in Sri Lanka were examined and sampled to culture and isolate non-tuberculous mycobacteria (NTM). A total of 35 mycobacteria (diseased=28, healthy=7) isolated from these fish were subjected to biochemical identification and Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP). Only 24 NTM isolated from diseased fish were identified at the species level, namely *Mycobacterium fortuitum* type I (n=7), *M. fortuitum* type II (n=3), *M. kansasii* type IV (n=5), *M. marinum* (n=8) and *M. chelonae* (n=1). Five of the seven NTM species isolated from apparently healthy fish were identified as *M. fortuitum* type I (n=2), *M. fortuitum* type II (n=1), *M. kansasii* type IV (n=1) and *M. marinum* (n=1). Irrespective of the NTM species involved, guppies most often showed skeletal deformities while chronic ulcers were common in goldfish and fighting fish. Histopathologically, granulomatous inflammation was minimum in guppies. All four NTM species isolated in this study are potentially zoonotic and it is necessary to implement appropriate biosecurity measures to prevent spread of these organisms.

Keywords: Mycobacteria, NTM, Piscine, PCR-RFLP

[*] Corresponding author. E-mail address: drad199@gmail.com

Introduction

Piscine mycobacteriosis is a sub-acute to chronic disease affecting more than 150 species of fresh, brackish and salt water fish (Decostere et al. 2004; Rhodes et al. 2005; Imajoh et al. 2013) . Although *Mycobacterium marinum*, *Mycobacterium fortuitum* and *Mycobacterium chelonnae* are the main etiological agents of this disease, there are several other slow and fast growing mycobacteria (*M. neoaurum*, *M. simiae*, *M. scrofulaceum*, *M. ulcerans, M. montefiorense*, *M. poriferae*, *M. shottsii*, *M. pseudoshottsii, M. avium* and *M. chesapeaki)* that have been implicated in infections in fish (Ucko et al. 2002; Gauthier and Rhodes 2009; Jacobs et al. 2009).

External clinical signs of the disease include progressive emaciation, chronic non-healing skin ulcers, abdominal swelling, scale protrusion, body deformation, faded pigmentation and anorexia (Lewis and Chinabut 2011). In addition, the affected fish may show melanotic foci on the skin and the spleen, organomegaly, peritonitis and visceral granuloma (Belas et al. 1995; Decostere et al. 2004; Jacobs et al. 2009). Early signs of mycobacteriosis may not be significant or specific for the disease, and visual clinical signs often do not develop until the infection becomes widely systemic (Beran et al. 2006). Non-tuberculous mycobacteria (NTM) in fish are transmitted mainly by consumption of contaminated feed, cannibalism of infected fish, through skin aberrations and vertically through the trans-ovarian route (Nigrelli and Vogel 1963; Hedrick et al. 1987; Harriff et al. 2007; Jacobs et al. 2009).

Because of the slow progression of the disease, younger fish infected with NTM show no external signs and the infection becomes more serious as the fish age or are stressed (Passantino et al. 2008). Piscine mycobacteriosis has gained more attention after the discovery of the zoonotic potential of some of the fish pathogenic mycobacteria, such as *M. marinum*, *M. fortuirum* and *M. ulcerans* (Huminer et al. 1986; Meyers et al. 1996; Ucko and Colorni 2005; Stinear et al. 2007). Routine diagnosis of mycobacteriosis has been based on the staining of tissue smears for acid-fast bacilli (AFB) and culturing of internal organs for mycobacteria. Looking for AFB in a smear is a quick technique (Tenover et al. 1993), but less sensitive and not reliable for accurate diagnosis as mycobacterium species are often isolated from smear negative samples and also there are more than 100 species of saprophytic and non-pathogenic mycobacteria present in various terrestrial and aquatic environments (Tenover et al. 1993; Decostere et al. 2004).

Therefore, accurate diagnosis of piscine mycobacteriosis requires the determination of the species involved. The isolation and identification of NTM species causing infections in ornamental fish in Sri Lanka are presented in this paper. Different organs of ornamental fish suspected of carrying NTM infections as well as apparently healthy fish from the same tanks were sampled to identify initial colonisation sites of NTM species.

Materials and methods

Collection of samples

Fresh water ornamental fish showing clinical signs suggestive of mycobacteriosis, and apparently healthy fish were collected from private pet shops and fish breeding farms located in Kandy, Kurunegala, Kaluthara and Polonnaruwa Districts of Sri Lanka from 2009-2013. Presence of chronic non-healing ulcers on the skin, faded pigmentation, emaciation and deformities in the body (mainly scoliosis) were used as the criteria to select diseased fish. During the study period, 55 guppies (*Poecilia reticulata* Peters 1859), 12 Siamese fighting fish (*Betta splendens* Regan 1910), ten Swordtails (*Xiphophorus variatus* Meek 1904), six Platies (*Xiphophorus maculatus* Günther 1866) and six goldfish (*Carassius auratus auratus* Linnaeus 1758), which showed at least one or more of the above clinical signs, and 30 apparently healthy guppies were sampled. After external examination, all fish were humanely euthanised using tricane methane sulphonate (MS-222) at a dose rate of 250 $mg.L^{-1}$ and subjected to a detailed necropsy. Internal lesions were recorded and squash preparations of the internal organs (kidney, liver, intestines, muscle) were stained with Zheil Neelsen (ZN) stain. At least 50 fields of each smear were examined to detect the presence of acid-fast organisms.

Histopathology

Depending on the size of the fish examined, whole fish or individual organ samples (kidney, liver) of selected fish showing signs typical of mycobacteriosis were fixed in 10 % phosphate buffered formalin for histopathological examination. These samples were processed following standard procedures and 5µm thick tissue sections were stained with ZN and Hematoxylin and Eosin (H and E) stains.

Isolation of NTM

Internal organs including liver, intestine, gonads and muscle tissues were sampled aseptically from each fish and placed in four sterile micro-centrifuge tubes for microbiological examination. Tissue samples for culture were decontaminated by incubating for 30 minutes with an equal volume of 1M NaOH (w/v) and washing with distilled water. Decontaminated organ samples were cultured separately on Ogawa egg medium in duplicate and incubated aerobically at ambient temperature and 37 °C for two months.

All samples were examined daily for seven days and then twice a week for rest of the period. Colonies were subjected to ZN stain and the acid alcohol fast colonies were sub-cultured on Ogawa egg medium. Identification was based on growth characteristics (i.e. slow or fast growth, pigment production and growth on MacConkey agar) and biochemical properties namely nitrate reduction, catalase production, tween-80 hydrolysis, urease production and NaCl tolerance.

Identification of NTM by PCR-RFLP

The DNA was extracted from *Mycobacterium* isolates (n = 35) by the boiling method. Briefly, a loopful of bacterial colonies was suspended in 500 µl of distilled water in a 1.5 ml sterile micro centrifuge tube and boiled for 10 min. After cooling at room temperature, the sample was centrifuged at 5000 g for 10 min and supernatant containing DNA was transferred into a new tube. The amplification of the 360 bp region of *rpoB* gene of mycobacteria was performed according to Lee et al. (2000) using the forward and reverse primers: 5'-TCAAGGAGAAGCGCTACGA-3; and, 3'-GGATGTTGATCAGGGTCTCG-5' (Sigma, St. Louis, MO, USA) respectively. PCR reaction mixture of 50 µl consisted of 10 µl of 5x reaction buffer, 28.8 µl of pyrogen free water, 50 pMol of each primer, 1 µl of 10 mM dNTP, 2 units of *Taq* DNA polymerase and 5 µl of template DNA. The PCR amplification profiles were: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min with a final extension for 7 min at 72 °C. After confirming the 360 bp amplicons by gel electrophoresis, the PCR products were digested with the restriction enzymes namely *Msp I* and *Hae III* by incubating for 1 h at 37 ºC. Thereafter, the restriction fragments were separated on 3 % high resolution agarose (Promega, USA) by electrophoresis and visualised under UV illumination. The interpretation of the RFLP pattern of the isolates was based on the algorithm described by Lee et al. (2000).

Results

Of the 89 diseased fish investigated, *Mycobacterium* spp. were isolated from one or more of the internal organs (spleen, liver, muscles, gonads, intestine) of 28 (29.2 %) fish that included 19 guppies, three Platies, two Siamese fighting fish, two Swordtails and two goldfish. Mycobacteria were most frequently isolated from the liver (84.6 %), spleen (69.2 %) and intestines (46.1 %) and less frequently from the muscles (19.2 %) and gonads (7.7 %). Faded pigmentation/skin discolouration (84.6 %) and emaciation (76.9 %) were the commonest external clinical signs observed in the fish from which Mycobacteria were isolated from internal organs, followed by body deformities (42.3 %) and skin ulcers (34.6 %). Body deformities (scoliosis) were observed only in guppies. In contrast, non-healing skin ulcers were less common in guppies (17.6 %) but frequently present in other species of fish (66.7 %), mainly goldfish and Siamese fighting fish. Mycobacteria were also isolated from 23.3 % of apparently healthy guppies with no external signs suggestive of mycobacteriosis, mostly from liver (42.8 %) and intestines (42.8 %) and less commonly from the spleen (28.5 %). Of culture-positive diseased fish (n=26), acid fast rods were observed microscopically in squash preparations of kidney and/or liver of 24 fish (92.3 %) that included 16 guppies (94.1 %), one Siamese fighting fish (50 %), two swordtails (100 %), three platies (100 %) and two goldfish (100 %) (Table 1). However, eight guppies, two Siamese fighting fish, three swordtails, one platy and one goldfish which showed clinical signs suggestive of mycobacteriosis were culture negative though they were positive for acid fast rods at microscopic examination of organ smears. Out of culture positive apparently healthy guppies, only 14.3 % had acid fast rods in ZN stained smears of internal organs.

Table 1. Distribution of non-tuberculous mycobacterium species (NTM) in various organs and tissues of ornamental fish

Fish Species	Lesions					Organs					Mycobacterium Spp
	Skin ulcer	Scoliosis	Skin discolouration	Emaciation	Acid fast organisms	Liver	Spleen	Intestines	Muscle	Gonads	
Guppy	-	+	+	+	+	+	+	-	-	-	*M. fortuitum* type II
(17/55)	-	+	+	+	+	+	+	-	-	-	*M. fortuitum* type I
(Poecillia reticulata)	-	+	+	+	+	+	+	-	-	-	*M. marinum*
	-	-	+	+	+	+	+	+	-	-	*M. fortuitum* type I + (NI)
	-	+	+	+	+	+	-	+	-	-	*M. kansasii* type IV
	-	+	+	+	+	+	-	-	+	+	*M. marinum+M. kansasii IV*
	+	-	+	+	+	+	+	+	+	-	*M. kansasii* type IV
	-	-	+	+	+	+	-	+	+	-	*M. fortuitum* type I
	-	-	+	+	-	-	+	+	-	-	*M. marinum*
	-	+	-	-	+	+	+	+	-	-	*M. marinum*
	-	+	+	-	+	+	+	+	-	-	*M. fortuitum* type I
	+	+	+	+	+	-	+	-	-	-	*M. marinum*
	+	+	-	-	+	-	+	-	+	+	*M. fortuitum* type II
	-	+	-	-	+	-	+	-	-	-	*M. marinum*
	-	+	+	+	+	+	+	-	-	-	*M. fortuitum* type I
	-	-	+	+	+	+	+	-	-	-	*M. chelonae*
	-	-	+	+	+	+	+	+	-	-	*M. marinum*
Apparently healthy	-	-	-	-	-	+	-	-	-	-	*M. kansasii type IV*
Guppy (7/30)	-	-	-	-	-	+	-	-	-	-	*M. marinum*
	-	-	-	-	-	+	+	-	-	-	*M. fortuitum* type I
	-	-	-	-	+	-	-	+	-	-	not identified
	-	-	-	-	-	-	-	+	-	-	*M. fortuitum* type I
	-	-	-	-	-	-	+	-	-	-	*M. fortuitum* type II
	-	-	-	-	-	-	-	+	-	-	NI
Fighting fish (2/12)	+	-	-	+	+	+	-	+	-	-	*M. fortuitum* type I
(Betta splendens)	+	-	+	+		+	+	-	-	-	*M. fortuitum* type II
Sword tail (2/10)											
(Xiphophorus	+	-	+	-	+	+	-	+	-	-	*M. kansasii* type IV
variatus)	-	-	+	-	+	+	-	-	-	-	not identified
Platies (3/6)	-	-	+	+	+	+	-	+	-	-	*M. kansasii* type IV
(Xiphophorus	-	-	+	+	+	+	+	-	-	-	NI
maculatus)	+	-	+	+	+	+	-	-	-	-	NI
Goldfish (2/6)											
(Carassius auratus)	+	-	+	+	+	+	+	+	-	-	*M. fortuitum* type I
	+	-	+	+	+	+	+	-	-	-	*M. marinum*

NI: not identified

Histopathological examination of infected fish revealed the presence of diffuse or organised/nodular granulomatous inflammation in varying degrees of infection in kidney, liver and spleen. Often, the lesions present in histological sections of guppies and Platys were diffuse in nature (Fig. 1a) whereas in goldfish more organised, granulomatous lesions were observed (Fig. 1b). The presence of acid fast rods in the tissues was confirmed by the ZN stain.

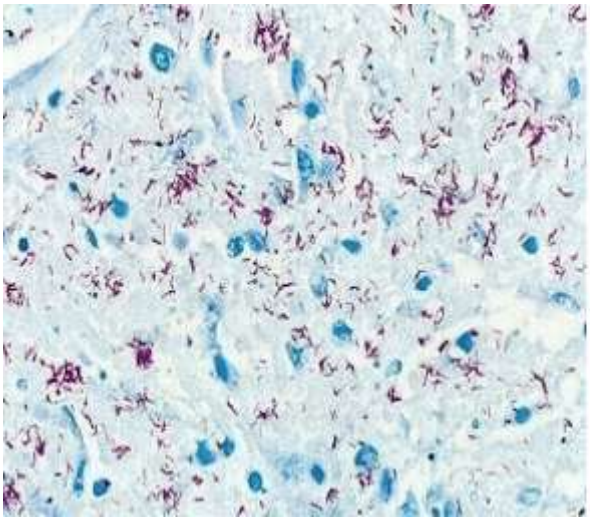

Fig. 1a. Histological section stained with ZN of a liver tissue of a guppy showing acid fast organisms (x1000)

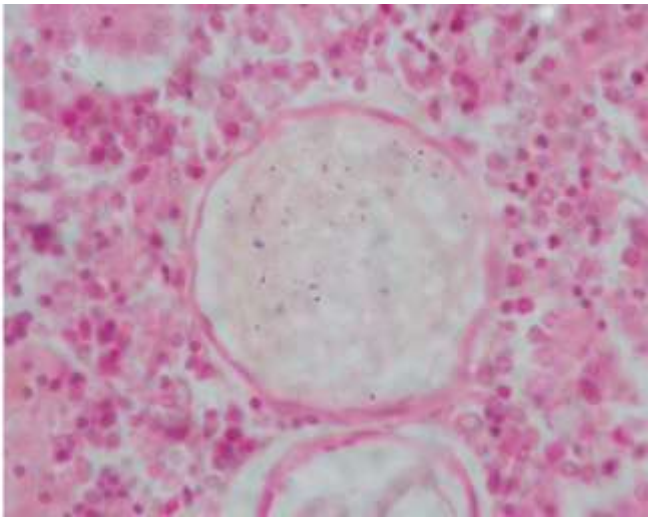

Fig. 1b. H& E stained section of a kidney of a goldfish showing more organised granulomatous lesions (X1000)

In order to identify the isolated mycobacteria, the present study used a combined approach. As such, different isolates were identified at the species level based on their phenotypic and biochemical properties as well as the RFLP pattern of the *rpoB* digests. All 28 isolates hydrolysed Tween 80 but none of the isolates were able to grow on LJ medium containing 5 % NaCl. Except for eight isolates, all other isolates were positive for catalase test. Ability to reduce nitrate to nitrite was observed only in 17 isolates. Of the 28 acid fast organisms isolated, only 24 (85.7 %) could be identified at the species level as follows: *M. fortuitum*, (catalase +ve, nitrate reduction +ve, tween 80 hydrolysis +ve and none pigmented colonies) type I (n=7), *and* type II (n=3), *M. kansasii* type IV (n=5, catalase +ve, nitrate +ve, tween 80 hydrolysis +ve, pigmented colonies), *M. marinum* (n=8, catalase -ve, nitrate reduction -ve, tween 80 hydrolysis +ve and pigmented colonies) and *M. chelonae* (n=1, catalase +ve, nitrate reduction -ve, tween 80 hydrolysis +ve, none pigmented colonies).

Phenotypic and biochemical profiles, and RFLP pattern of the rest of the isolates (14.3 %) could not be matched to those of *Mycobacterium spp*. known hitherto. Co-infection by different species of *Mycobacterium* was observed in two guppies.

The percentage of isolation of different species of mycobacteria from apparently healthy guppies was: *M. fortuitum* type I (28.6 %), *M. fortuitum* type II (14.3 %), *M. kansasii* type IV (14.3 %), *M. marinum* (14.3 %) and unidentified (28.5 %). The species of *Mycobacterium* isolated in the present study, together with their fish hosts are shown in Table 1.

PCR amplification of the *rpoB* gene of all *Mycobacterium* strains isolated in the present study (n=35) yielded the expected 360 bp fragment. However, only 29 (82.8 %) isolates provided comparable RFLP pattern to a known species or strains (Fig. 2).

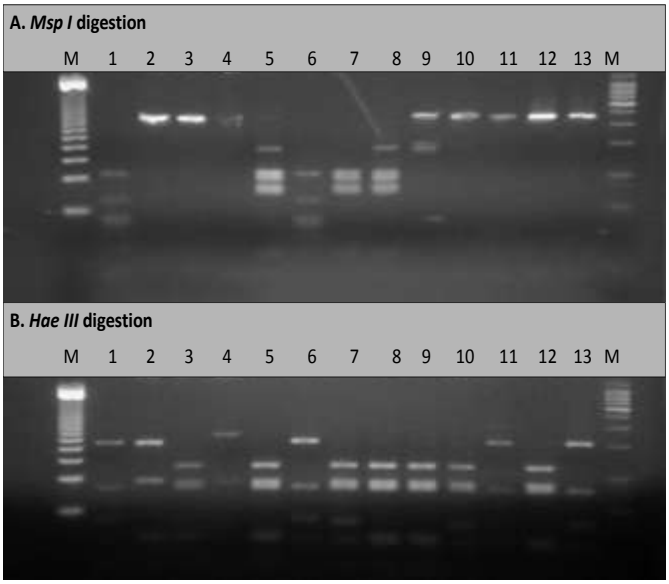

Fig. 2. *Msp*I (A) and *HaeIII* (B) restriction enzyme digestion patterns *of rpoB* gene of NTM. Lane M: DNA size marker (50bp ladder). Lane 1 and 6 (Msp I:100, 60, 40 and Hae III: 200, 80)- *M. marinum.* Lane 5 and 8 *(MspI:* 175, 100, 70, and *Hae III:* 120, 90, 80)- *M. fortuitum I.* Lane 2,3, 9,10,11,12 and 13- inconclusive as the *MspI* digestion failed and hence was repeated (*MspI:* lane 2, 3, 4, 9. 10, 11, 12, 13 of image A contained the 360bp amplicon of *rpoB* -not digested by MspI, *Hae III*: lane 2, 4, 11, 13: 200, 80; lane 3, 9, 10 and 12: 120, 90, 80).

Discussion

Of over 20 *Mycobacterium* species reported to be isolated from fish, three species namely *M. marinum*, *M. fortuitum* and *M. chelonae* are known to cause clinically significant diseases in fresh water ornamental fish (Gauthier 2015). Results of the present study confirmed that these three NTM species were also involved in mycobacteriosis in freshwater ornamental fish in Sri Lanka and this is the first report of the infection among ornamental fish in the country. Frequency of isolation of *M. marinum* and M. *fortuitum* from ornamental fish was similar and higher than that of *M. chelonae*. A recent study that examined the occurrence of *Mycobacterium* species in fish from Swedish wholesalers also reported a high prevalence of *M. marinum.*

However, the rate of isolation of *M. fortuitum* in their study was lower than ours (Hongslo and Jansson 2016). Pate et al. (2005) reported an approximately equal occurrence of *M. fortuitum*, *M. gordonae* and *M. marinum* in aquarium fishes in Slovenia. Another study conducted in Italy reported that the *M. fortuitum* and *M. chelonae* were more prevalent than *M. marinum* (Zanoni et al. 2008). Altogether, these results indicated that the frequency of isolating these three NTM species may vary from country to country; undoubtedly, they are the common NTM species affecting freshwater ornamental fish.

Moreover, *M. kansasii*, which is not commonly designated as a fish pathogen, was also isolated from diseased as well as from apparently healthy fish in this study. *M. kansasii* is a slow growing NTM species which has been commonly isolated from tap water and soil. Since tap water is frequently used in aquaria, growth of *M. kansasii* may represent host colonisation (Johnston et al. 2017) or may cause opportunistic infection in immunocompromised fish. Another recent study that examined apparently healthy aquarium fish from pet shops for NTM has also reported the isolation of *M. kansasii* (Kušar et al. 2016). Infrequent isolation of *M. kansasii* from diseased and apparently healthy fish warrants further studies to confirm its role as a fish pathogen. Despite the different growth rates and requirements, there was no considerable variation observed in the site of colonisation of the above four species. Further, it was observed that the clinical presentation was based on the species of the host and not on the species of the NTM involved. Accordingly, in this study the guppies most often showed skeletal deformities irrespective of the *Mycobacterium* species involved. Further, the histopathological evidence suggests that granulomatous inflammation is minimum in guppies.

In contrast, several studies have reported systemic granulomatous lesions as the most remarkable finding in guppies which were infected by *M. gordonae* (Sakai et al. 2005). Gomez (2008) has reported the presence of both diffuse and nodular granulomatous lesions in various fresh water ornamental fish affected with NTM. Findings of that study indicated that nodular lesions could be seen in advanced cases. Although described as a chronic process, the disease may evolve without gross lesions such as gray to white nodules in multiple organs or microscopic or histopathologic evidence of granulomatous disease that is generally associated with mycobacteriosis in fish. The present histopathological study shows that gross pathology, where nodules are assumed to be indicative of tubercular disease in fish, can be useful only when the state of disease is advanced and when the size of the fish allows a suitable necropsy. Among NTM species, *M. marinum* is known to have high zoonotic potential. *M. marinum* was isolated from seven diseased fish and one apparently healthy fish. This organism is known to cause soft tissue infections particularly in fish handlers (Huminer et al. 1986; Lewis et al. 2003; Ucko and Colorni 2005). *M. kansasii* is also capable of causing cutaneous infections resembling sporotrichosis in humans, particularly in immunocompromised individuals or patients with skin pathologies (Breathnach et al. 1995; Fleming and Keystone 2017). *M. chelonae* and *M. fortuitum* have also emerged as zoonotic organisms and reported to cause soft tissue and bone infections following medical and surgical interventions (Wang and Pancholi 2014).

In general, several fish pathogenic mycobacteria cause non-pulmonary tuberculosis mainly in immunocompromised individuals. As the proportion of immunocompromised individuals (i.e. cancer patients, organ transplant patients, HIV patients) and the aging population is ever increasing, it is important to follow necessary biosecurity measures to prevent the establishment of these pathogens in fish and water resources. Identification of the species of NTM affecting fish is important in several aspects. As mentioned previously, a large number of NTMs are non-pathogenic inhabitants of water and soil. Thus, it is important to confirm the isolated organism as a known fish pathogen.

In addition, most NTM species are resistant to many commonly used antiseptics and antibiotics and these resistances are genetically linked to the species or to the strain. Identifying the species of the *Mycobacterium* is important for selecting appropriate treatment or decontamination procedures in aquaculture facilities. RFLP patterns of *rpoB* gene made it possible to identify more *Mycobacterium* isolates to the species level within a limited time compared to phenotypic tests. When pure culture is available, identification of species can be done within one working day by RFLP-PCR, whereas biochemical tests require 2-3 weeks. Routine conventional tests were unable to differentiate the species within the *M. fortuitum* group, as well as in the group containing *M. chelonae* and *M. abscessus* (Lee et al. 2000). Moreover, unlike conventional methods which require excessive growth of mycobacteria, RFLP-PCR can be performed with a single colony.

It is apparent that the PCR-RFLP is a reliable and rapid method of identifying NTM species but the technique has several limitations which could hamper correct identification. Presence of single nucleotide polymorphism in a restriction enzyme recognition site, may affect the digestion and will result in non-comparable RFLP patterns. The most challenging situations encountered in the present study were the discrimination of certain band sizes (eg. 95 bp from 90 bp and 100 bp from 105 bp) that are critical in identifying the species. Obtaining an appropriate separation on very close band sizes (eg. 80 bp and 70 bp) was also challenging in certain instances. Of the 35 *Mycobacterium* isolates, 29 were identified by PCR-RFLP at the species level. The RFLP-pattern of the five remaining isolates did not match any known *Mycobacterium* species and this might represent the mycobacteria hitherto undescribed or certain other acid fast bacilli. Some difficulties were encountered with the interpretation of three of the isolates due to the presence of non-specific gel bands, perhaps due to partially digested gene fragments.

Conclusion

NTM infections are present among ornamental fish in Sri Lanka. The pathology of the infections seems to be more related to the species of the host affected rather than that the species of *Mycobacterium* involved. NTM species detected in ornamental fish in the present study are potentially zoonotic. Therefore, it is necessary to implement appropriate biosecurity measures to prevent further spread of these organisms.

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