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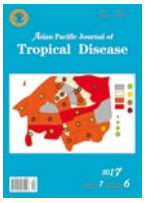

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Detection of *Mycobacterium marinum* in clinically asymptomatic Siamese fighting fish (*Betta splendens*) from ornamental fish shops in Chiang Mai Province, Thailand

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ABSTRACT

Objective: To detect *Mycobacterium marinum* (*M. marinum*) infections in healthy Siamese fighting fish from ornamental fish shops in Chiang Mai, Thailand.
Methods: *Mycobacterium* spp. were isolated from 380 internal organs of healthy Siamese fighting fish using Löwenstein-Jensen and Middlebrook 7H10 culture media. A 924-bp DNA fragment from mycobacterial 16S rRNA was amplified and digested with *Ban*I and *Apa*I restriction enzymes to yield unique restriction patterns for each mycobacterial specie.
Results: Thirty-five mycobacterial isolates (8.42%) were recovered from 380 Siamese fighting fish; 21 isolates (5.5%) and 11 isolates (2.29%) were identified as *M. marinum* and *Mycobacterium chelonae*, respectively.
Conclusions: The results demonstrated the presence of *M. marinum* zoonotic bacterial pathogens in healthy Siamese fighting 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

Mycobacterium marinum (*M. marinum*) was first isolated in the USA in 1926[1]. It is a slow-growing, nontuberculous mycobacterium found in both freshwater and saltwater worldwide. *M. marinum* is an opportunistic bacterium that may cause a chronic granulomatous skin disease in humans called 'fish tank granuloma' or 'aquarium granuloma'. *M. marinum* is transmitted to the human body through wounds or skin abrasions; it primarily causes skin lesions at the cooler parts of the body, especially the extremities[2]. A study of nontuberculous mycobacterial (NTM) infections of the skin showed that 81.8% of NTM skin infections were caused by *M. marinum*, and most patients had hobbies related to fish and water[3]. *M. marinum* infection causes a red-to-violaceous plaque or nodules, usually in a sporotrichoid pattern, although extensive deep skin lesions may occur. Osteomyelitis and tenosynovitis can be observed in some cases[4]. The recommended chemotherapeutic regimens for *M. marinum* infection in humans include rifampicin plus ethambutol, clarithromycin, rifampicin, ethambutol and amikacin, or trimethoprim/sulfamethoxazole, with variable susceptibility to minocycline, doxycycline, and streptomycin[5,6].

Siamese fighting fish (*Betta splendens*) belongs to the genus *Betta* and are among the most important ornamental fish in terms of its body coloration, shape, numbers and notable commercial value[7]. According to the Department of Fisheries of Thailand, more than 14 million fighting fish were exported in 2010, with a value of more than 10 million USD[8].

This study used a conventional culture procedure to isolate mycobacteria, and used primers specific to 16S rRNA, including *Ban*I and *Apa*I restriction enzymes, to identify *M. marinum* species. The aim of this study was to detect *M. marinum* in healthy Siamese fighting fish from ornamental fish shops in Chiang Mai, Thailand.

2. Materials and methods

2.1. Sample collection

A total of 380 Siamese fighting fish were collected from three major ornamental fish shop areas – Kamthieng Market, Buak Haad Park and Pratu Haiya – in Chiang Mai, Thailand from April to June 2014. Fish were euthanized by using an overdose (250 mg/L) of tricaine methanesulfonate (MS222, Sigma–Aldrich) buffered to pH 7.2 with sodium bicarbonate. Internal organs (100 mg) from each fish were collected under aseptic conditions and kept at –20 ˚C until bacteriological examination. This study was conducted

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in accordance with the protocols approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, Chiang Mai University (No. S4/2555).

2.2. Bacterial culture

Internal organs were homogenized with phosphate buffered saline and decontaminated with NaOH and *N*-acetyl-L-cysteine solution according to a procedure described previously[9]. Mycobacteria were cultured on Löwenstein–Jensen medium and Middlebrook 7H10 agar (Becton, Dickinsonand Co., Franklin Lakes, NJ, USA) for 8 weeks at 30 ℃. Colonies that showed specific characteristics of mycobacteria, *i.e.*, white to cream in color and a rough to smooth surface, were selected and stained with acid-fast stain. Acid-fast bacilli were observed under a light microscope.

2.3. Molecular techniques for M. marinum

2.3.1. DNA extraction

DNA was extracted by boiling method, as previously described by Vary *et al.*[10]. Briefly, mycobacterial colonies on agar were suspended in 200 mL of 0.2 mol/L NaOH. The cells were lysed at 120 ℃ for 10 min, and supernatant was collected and kept at –20 ℃ until further study.

2.3.2. Polymerase chain reaction (PCR) and restriction enzyme analysis

PCR was carried out using genus-specific primers designed from a conserved region of 16S rRNA for mycobacterium – T39 (5′-GCG AAC GGG TGA GTA ACA CG-3′) and T13 (5′-TGC ACA CAG GCC ACA AGG GA-3′) – to produce a 924-bp PCR product, according to the method previously described by Talaat *et al.*[11]. Total DNA was amplified by PCR containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.8 mmol/L deoxynucleotide triphostphate, 1.25 IU of *Taq* DNA polymerase (RBC Bioscience®, Taiwan) and ultrapure water to a final volume of 25 μL.

Touch-down amplification was performed with an initial step of 95 ℃ for 2 min, followed by 10 cycles of 95 ℃ for 30 s, with annealing temperatures decreasing from 68 to 58 ℃ for 56 s; the subsequent 25 cycles were maintained at 58 ℃ with annealing step for 56 s. The final extension step was at 72 ℃ for 10 min. A negative control was prepared with sterile water to check acontamination. PCR products were sequenced to confirm amplicon identity.

The PCR product of 924 bp in size was analyzed by restriction enzyme digestion with *Ban*I and *Apa*I restriction enzymes (New England Biolabs). The digestion profiles of *M. marinum* with *Apa*I yielded three DNA fragments of 677, 132 and 115 bp, while the *M. marinum* product remained intact with *Ban*I[11].

2.3.3. Agarose gel electrophoresis

The amplified PCR products and digested DNA samples were loaded on 2% (w/v) agarose gel containing 0.05 μL/mL^{-1} of RedSafe™ (iNtRON Biotechnology Inc., Korea) and 1× tris-acetate-ethylene diamine tetraacetic acid buffer (pH 8.0) in an electrophoresis system. A 100-bp ladder (GeneDirex®, Taiwan) was used for size determination and photographed using the gel documentation system.

3. Results

After 8 weeks of incubation, 47 bacterial isolates were recovered from 380 samples; of these isolates, 35 had acid-fast characteristics, of which 28 were recovered from Löwenstein–Jensen medium and 7 were recovered from Middlebrook 7H10 medium. A 924-bp PCR product was amplified from 32 (8.42%) samples using T39 and T13 primers (Figure 1). As shown in Figure 2, two patterns of DNA fragments were observed after digestion with *Ban*I and *Apa*I restriction enzymes: a) a 924-bp fragment, and 677-, 132- and 115-bp fragments when digested with *Ban*I and *Apa*I restriction enzymes, respectively, indicated *M.marinum*; b) 562- and 362-bp fragments, and 812- and 112-bp fragments when digested with *Ban*I and *Apa*I restriction enzymes, respectively, signified *Mycobacterium chelonae* (*M. chelonae*). From the internal organs of 380 fighting fish, 21 samples (5.5%) *M. marinum* and 11 samples (2.9%) *M. chelonae* were isolated and identified.

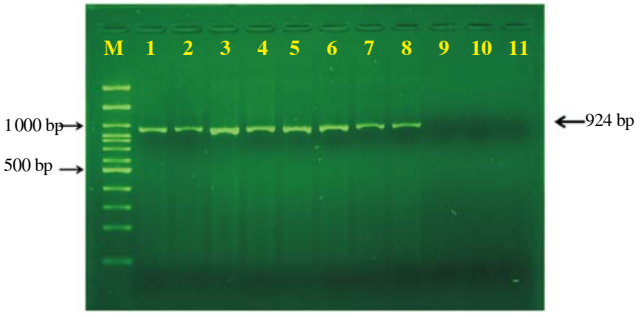

Figure 1. Agarose gel electrophoresis of PCR assay for the identification of 16S rRNA of mycobacterium using theT39/T13 primers.
Lane M: DNA marker; Lane 1: Positive control; Lanes 2–8: Positive isolates; Lanes 9 and 10: Negative isolates; Lane 11: Negative control.

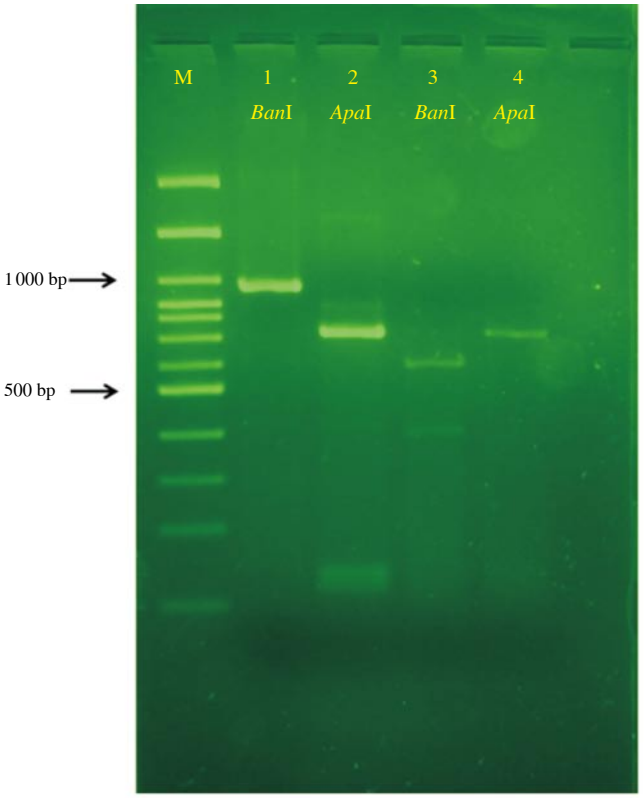

Figure 2. Restriction enzyme analysis of amplified 16S rRNA of mycobacterium digested with restriction enzymes *Ban*I and *Apa*I.
Lane M: DNA marker; Lane 1: *M. marinum* DNA digested by *Ban*I (924 bp); Lane 2: *M. marinum* DNA digested by *Apa*I (677 bp, 132 bp and 115 bp); Lane 3: *M. chelonae* DNA digested by *Ban*I (562 bp and 362 bp); Lane 4: *M. chelonae* DNA digested by *Apa*I (812 bp and 112 bp).

4. Discussion

Mycobacteriosis is a significant bacterial infection of fish and one of the more common chronic bacterial diseases of pet fish. Infections have been reported worldwide from various species of saltwater

and freshwater fish in both wild and aquatic environments[12]. *M. marinum, M. fortuitum*, and *Mycobacterium 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*)[13-15].

In our study, the internal organs of clinically asymptomatic Siamese fighting fish from ornamental fish shops were used to identify *M. marinum*. The results showed that the recovery rate of *Mycobacterium* spp. from Löwenstein–Jensen medium (28 samples) was higher than from Middlebrook 7H10 medium (7 samples). However, previous reports indicated no significant difference in the recovery rates from these culture media[16,17]. Morover, conventional bacterial culture methods used to diagnose *Mycobacterium* spp. are slow and rely only on phenotypic characteristics, which 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[18] and PCR results showed that 91.43% (32/35) of the acid-fast bacilli grown on culture media were *Mycobacterium* spp.

DNA amplification from the highly conserved region of 16S rRNA, together with a digestion step with *Ban*I and *Apa*I restriction enzymes, is very useful for identifying *M. marinum*, since this method yields a distinct DNA fragment pattern[19]. Our results showed that 5.5% of clinically healthy Siamese fighting fish carried *M. marinum*, which was higher than what was reported in a study based on phenotypes and random amplified polymorphic DNA (RAPD) in Malaysia[20]. However, other molecular techniques have also been shown to help with early detection and preventing the spread of this highly contagious bacterium. Salny[21] developed qPCR, which is a rapid, sensitive and specific method for detecting and quantifying *M. marinum* directly from tissue in approximately 6 h.

In conclusion, this study showed that fighting fish (*Betta* spp.) may play a role as an NTM infection source for risk groups, such as fishery professionals or ornamental fish hobbyists. Exposure to contaminated water and superficial cuts or abrasions of the skin are two major risk factors of *M. marinum* infection in non-immunocompromised patients[22,23]. Ornamental fish owners and others with high risk of *M. marinum* infection should be educated about the zoonotic risk associated with handling aquarium fish and the aquarium environment, including how to protect themselves from infection.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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