Original Article

Mycobacterium marinum and Mycobacterium fortuitum infections in Siamese fighting fish, Betta splendens (Regan), in Thailand

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Abstract

Mycobacterium marinum and Mycobacterium fortuitum are important causative agents of mycobacteriosis in fish worldwide. Moreover, M. marinum can cause granulomatous skin lesions in humans. The objectives of this study were to determine the prevalence of Mycobacterium infections in Siamese fighting fish, Betta splendens (Regan), in Thailand and to evaluate the pathogenicity of the Mycobacterium isolates in goldfish. In the prevalence study, 190 Siamese fighting fish (15 moribund and 175 clinically healthy fish), collected from culture farms and ornamental fish markets in Thailand, were included and examined. Mycobacterium spp. were isolated from the kidneys, spleen, liver and gills. The isolates were then identified on the basis of morphological, biochemical characteristics and the analysis of a partial 16S rRNA gene sequence. The prevalence of Mycobacterium infections in this study was 15/15 (100%) in the moribund fish and 50/175 (25.71%) in the clinically healthy fish. In the pathogenicity study, 150 clinically healthy goldfish were used to test for the 2 isolates (Mycobacterium marinum KKVB0901 and Mycobacterium fortuitum KKVB0926). Results from the test showed that both isolates were pathogenic to experimental goldfish.

Keywords: *Mycobacterium marinum*; *Betta splendens;* Siamese fighting fish; Fish disease

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Introduction

Mycobacterium marinum and *Mycobacterium fortuitum* are known as important causative agents of mycobacteriosis in many fish species worldwide including ornamental fish (Beran *et al*., 2006; Zanoni *et al*., 2008; Gauthier and Rhodes, 2009). Fish mycobacteriosis is a chronic, progressive disease affecting many organs of fish such as eyes, gills, visceral organs, muscles, and fins (Gauthier and Rhodes, 2009) and may result in a significant decrease in fish production especially a reduction in the market value of ornamental fish (Novotny *et al*., 2010). The affected fish often have granulomatous inflammation, a specific clinical manifestation, in various organs especially the kidney, digestive tract, liver and spleen (Novotny *et al*., 2010); however, they also often have non-specific clinical signs including abnormal behavior, scale loss, pigment changes, spinal defects, ascites and emaciation (Gauthier and Rhodes, 2009). In addition, *M. marinu*m and *M. fortuitum* can also be found in clinically healthy ornamental fish (Beran *et al*., 2006).

M. marinum and *M. fortuitum* are also zoonotic agents causing mycobacteriosis in humans. *M. marinum* infections in humans have been well documented (Feng *et al*., 2011; Bonamonte *et al*., 2013; Slany *et al*., 2013; Babamahmoodi *et al*., 2014). People at risk are professional fish handlers and aquarists. The infected cases usually develop cutaneous granulomas, commonly known as "fish tank granuloma" or "swimming pool granuloma" (Wu *et al*., 2012; Slany *et al*., 2013). Although infections with *M. marinum* or *M. fortuitum* are not a cause of death, severity may be seen in immune-compromised cases probably resulting in invasive infections and serious complications (Tamaki *et al*., 2012; Flondell *et al*., 2013).

The siamese fighting fish (*Betta splendens*) is an economically important fish for aquaculture in Thailand. According to a report of Inland Aquatic Animal Health Research Institute, Thailand in 2010, the fish were exported with a value over 12 million USD. This value had increased more than 10 times compared with that in 2009. More interestingly, Siamese fighting fish can be used as a biological control of an *Aedes aegypti* larva, a vector of dengue disease that is found in tropical and subtropical regions around the world (Cavalcanti *et al*., 2009; de Oliveira Lima *et al*., 2010; Paiva *et al*., 2014). The prevalence of mycobacterium infections in ornamental fish or fresh water fish varies from as low as 1.7% in one study (Mrlik *et al*., 2012) to as high as 41.7% in another study (Slany *et al*., 2014) and depends on methods of bacterial detection (Slany *et al*., 2014). Although the prevalence of *Mycobacterium* infections in Siamese fighting fish has been reported, little is known about the pathogenicity of *Mycobacterium* spp. isolated from Siamese fighting fish.

The objectives of the study were to determine the prevalence of *Mycobacterium* spp. in moribund and clinically healthy Siamese fighting fish collected from culture farms and ornamental fish markets in Thailand and to evaluate the pathogenicity of the isolated *Mycobacterium* spp., in goldfish, *Carassius auratus* (L.).

Materials and Methods

***Sample collection and bacteria isolation*:** In this survey, 15 moribund and 175 clinically healthy Siamese fighting fish were randomly collected from 5 culture farms and 3 ornamental fish markets in Khon Kaen, Ratchaburi and Bangkok provinces, Thailand. *Mycobacterium* spp., were isolated from the kidneys, spleen, liver and gills of each fish. The small pieces of each tissue mentioned above were homogenized with 4% NaOH for 10 min and then each homogenized solution was inoculated on 1% Ogawa medium (Nissui seiyaku, Japan), Middlebrook 7H10 agar with OADC (Becton Dickinson, USA), and BHI agar (Becton Dickinson, USA) by a loop. In addition, each tissue was also inoculated on BHI agar without treatment with 4% NaOH for detection of other bacteria. All samples were incubated at 25 °C for 2 months and then visible colonies were purified. In addition, other bacteria were also been isolated and identified by biochemical test.

Histopathology: The gills, spleen, kidneys, heart and liver from the moribund fish were fixed in 10% phosphate-buffered formalin solution, and later the gills were decalcified with 10% EDTA solution. The fixed samples were routinely embedded in paraffin and sectioned at 3 to 5 µm. The sections were stained with Haematoxylin and Eosin (H & E), Giemsa, and Ziehl Neelsen.

Mycobacterium identification: Morphological and biochemical characteristics: 63 out of 81 strains isolated from 60 infected fish were selected at random and were used for bacterial identification. They were classified according to a manual of clinical microbiology (Herbert and Robert, 1985), Bergey's manual of systematic bacteriology (Sneath *et al*., 1986) and Cowan and Steel's manual for the identification of medical bacteria (Barrow and Feltham, 1993). Morphologies of the isolated bacteria were observed on 1% Ogawa medium and Middlebrook 7H10 agar. Motility, shape, Gram-stain, acid-fast, and the growth on Middlebrook 7H10 agar containing 5% NaCl were examined as described by Herbert & Robert (1985) and Barrow and Feltham (1993). Pigmentation, arylsulfatase reduction, nitrate reduction, degradation of PAS (para aminosalicylic acid), inhibition by picric acid, urease and tween 80 hydrolysis were tested by commercial standard kits (Kyokuto seiyaku industry, Japan) that can differentiate 16 different species in the genus *Mycobacterium*.

A partial 16S rRNA gene sequence: Two strains (KKVB0901 and KKVB0926) from different kinds of biochemical characteristics used for the identification were selected at random for comparative analysis of a partial 16S rRNA gene sequence. The partial 16S rRNA gene was amplified and sequenced using PCR employing six prokaryotic 16S rRNA universal primers as described by (Kageyama *et al*. 2004) (Table 1). The 50 µL reaction mixtures contained 1.2U Taq DNA polymerase (Promega, USA), 1.5 mM MgCl2, 0.25 mM deoxynucleotide triphosphate, 0.25 µM each primer such as forward 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1542R (5'-AGGAGGTGGATCCAGCC-3') and

one bacterial colony of the strains in the buffer supplied with a commercially available Promega TaqTM. Amplifications were performed by a PCR thermal cycler (TaKaRa, Japan) and the following amplification conditions were used: 1 cycle of 94 °C (4 min), followed by 40 three-step cycles of denaturizing at 94°C (1 min), annealing 60 °C (1 min), extension at 72 °C (2 mins), and a final extension cycle at 72 °C (5 min). The PCR products were electrophoresed on a 2% agarose gel stained by ethidium bromide and illuminated with UV light. Each PCR product was recovered and purified by PCR purification kit (ExoSAP-IT, Ambion, CA). Briefly, 5 µl of PCR product was mixed into 2 µl of ExoSAP-IT and incubated at 37°C (15 min) and inactivate ExoSAP-IT by heating to 80°C (15 min). Then, 1 µl of PCR product was placed into 6 vial (0.2 ml vial), containing 14 µl of DNase free water, 4 µl of BigDye Terninator V1.1 Cycle Sequencing Kit (Applied Biosystems, USA), and 1 µl (3.0 pmol/µl) of each primers, such as 8F, 520F, 926F, 691R, 1100R and 1542R. Each vial consisted of only one kind of primer. The product was placed in a PCR thermal cycler and the following amplification conditions were used, 1 cycle of 98°C (4 mins), followed by 25 three-step cycles of denaturizing at 98°C (10 secs), annealing 50°C (7 secs), and extension at 60°C (4 mins). Then the product was precipitated and dehydrated. They were sequenced using an automated sequencer (3100 Avant Genetic Analyzer, ABI PRISM) according to standard manufacturers' protocols. Alignment of the nucleotide sequences and homology analysis were analyzed using SeqMan software (DNAstar) and BLAST programs (http://www.ncbi.nlm.nih.gov/blast).

Table 1 Six prokaryotic 16S rRNA universal primers used for the identification of *Mycobacterium* spp., in this study

Primer		
8 F	5′- AGAGTTTGATCCTGGCTCAG-3′	Forward
520 F	5′- CAGCAGCCGTAATAC -3′	Forward
926 F	5′- AAACTCAAAGGAATTGACGG -3′	Forward
691 R	5′- TCTACGCATTTCACC -3′	Reverse
1100 R	5′- GGGTTGCGCTCGTTG -3′	Reverse
1542 R	5′- AGGAGGTGGATCCAGCC -3′	Reverse

Pathogenicity test: A total of 150 clinically healthy goldfish, *Carassius auratus* (L.), (3.25-4.10 g in body weight and 17-20 cm in total length) were used for experimental infection. The goldfish is a suitable infection model and often used for pathogenicity study (Talaat *et al*., 1998; Choe *et al*., 2017; Jin *et al*., 2019) and bacterial infection need to investigate before using instead of the Siamese fighting fish that may be naturally infected. The cumulative mortality observation requires a long observation period because the bacterial infection might grow slowly and because of unstable disease formation. Therefore, the experiments are necessary to use animals in sufficient amount. These fish were fed pellets on a daily basis and then were divided equally into 5 groups (4 experimental groups and 1 control group, n = 30/group). *Mycobacterium marinum* KKVB0901 was adjusted with 0.85% NaCl to 2.83 x 105 and 2.83 x 103 CFU mL-1 (for group 1, the higher dose of *M. marinum* and group 2, the lower dose of *M. marinum*, respectively) and *M. fortuitum* KKVB0926 was adjusted with 0.85% NaCl to 3.49 x 105 and 3.49 x 103 CFU mL-1 (for group 3, the higher dose of *M. fortuitum* and group 4, the lower dose of *M. fortuitum*, respectively). In experimental infection groups, each fish was intramuscularly injected with the bacterial suspension according to group assignments described above at a dose of 0.1 mL per fish. In control group, each fish was intramuscularly injected with 0.1 mL of 0.85% NaCl.

All groups were observed for cumulative mortalities and bacteria were re-isolated from moribund fish at various times post-infection. At the end of the experiment, 210 days after experimental infection, the surviving fish were euthanised followed IACUC methods by appropriately trained personnel with sedative agents: tricaine methane sulfonate, then rapid submerging of the fish in 2 to 4◦C for 10 minutes following cessation of operculum movement. Finally, the bacteria were re-isolated. These sample collections and investigations were performed for reasons of medical indication. The experiments were conducted in strict accordance with the recommendation in the Japanese guidelines and regulations for scientific and ethical animal experimentation (the 3R principal) and followed the previous methods of Weerakhun *et al*. (2008).

Results

Prevalence: The prevalence of *Mycobacterium* infections was 15/15 (100.0%) in the moribund fish but was 50/175 (25.7%) in the clinically healthy fish. Almost all fish were infected with *M. marinum* and *M. fortuitum*. Co-infections with these bacteria accounted for 60.0% in the clinically healthy fish, include *Aeromonas hydrophila*, *Pseudomonas* spp., *Streptococcus* spp. and *Edwardsiella tarda*. However, the *Mycobacterium* infections in the moribund fish were identified as only *M. marinum*.

Clinical signs and gross findings: The affected fish had lethargy, anorexia, emaciation, and abdominal distension (Fig 1a). Coelomic membrane edema with ascites was seen (Fig 1b). Inflammation of several internal organs with white nodules was generally found including the kidneys, spleen, and liver. Hepatomegaly, splenomegaly, and kidney enlargement were observed (Fig 1c).

Histopathological findings: Acid-fast bacilli stained with Ziehl-Neelsen were found in the granulomas and parenchyma of examined tissues (Fig 2a). Disseminated granulomatous hepatitis, granulomatous splenitis and granulomatous nephritis were observed. All granulomas were classified as soft tubercle-type without caseous necrosis in the center and without the hard periphery so that fibroblast and

multi-layer of epitheloid cells could be found at the border of the granuloma (Fig 2b).

Identification: According to the morphological and biochemical characteristics, the bacterial isolates could be classified into 2 different types. The first type consisted of 39 strains that were Gram-positive, acid-fast, non-motile and photochromogenic. All of these strains were slowly growing at 15 to 32 ˚C with an optimum temperature of 25 ˚C on Middlebrook 7H10 agar showing smooth colony. The colony did not grow on 5% NaCl Middlebrook 7H10 agar. They were negative for the reduction of nitrate and degradation of PAS. The isolated bacteria resisted picric acid inhibition and were positive for reduction of arylsulfatase (at day 14), urease, Tween 80 hydrolysis (at day 5), and semi-quantitative catalase. These strains were classified as *Mycobacterium marinum* (Table 2). The second type consisted of 24 strains that were also Gram-positive, acid-fast and non-motile; however, these strains were non-photochromogenic and had rapid growth at 15 to 42˚C with an optimum temperature of 25-37˚C on Middlebrook 7H10 agar and were rough colony. They can grow on 5% NaCl Middlebrook 7H10 agar. They were positive for all the same biochemical tests used identified *Mycobacterium* spp. These strains were classified as *Mycobacterium fortuitum* (Table 2).

The electrophoresis figure showed specific primer pairs amplified the expected size PCR products from two isolates KKVB0901 and KKVB0926 compared other *Mycobacterium* spp. (Fig 3). The products of approximately 1400 bp length of a partial 16S rRNA gene of both isolates were analyzed. The isolates were identified as *Mycobacterium marinum* and *Mycobacterium fortuitum*, respectively. This identification was in accordance with their morphological and biochemical characteristics.

Pathogenicity test: The cumulative mortality of the goldfish injected with M. marinum KKVB0901 and *M. fortuitum* KKVB0926 depended on the dose (Fig 4). However, no fish died within 90 days after the experimental infection. The experiments were continually observed for 210 days. The fish injected with a higher dose of *M. marinum* KKVB0901 died at 100.0% within 156 days, whereas the lower dose died at 100.0% on day 197 after experimental infection (Fig 4a). The fish injected with higher dose and lower doses of *M. fortuitum* KKVB0926 died at 88.0% and 72.0%, respectively within 210 days after the experimental infection (Fig 4b). Results from re-isolation of the bacteria showed that 100% of the experimental fish were positive for both *M. marinum* and *M. fortuitum*. In contrast, no fish in the control group died and all fish in this group were negative for the bacterial culture and isolation.

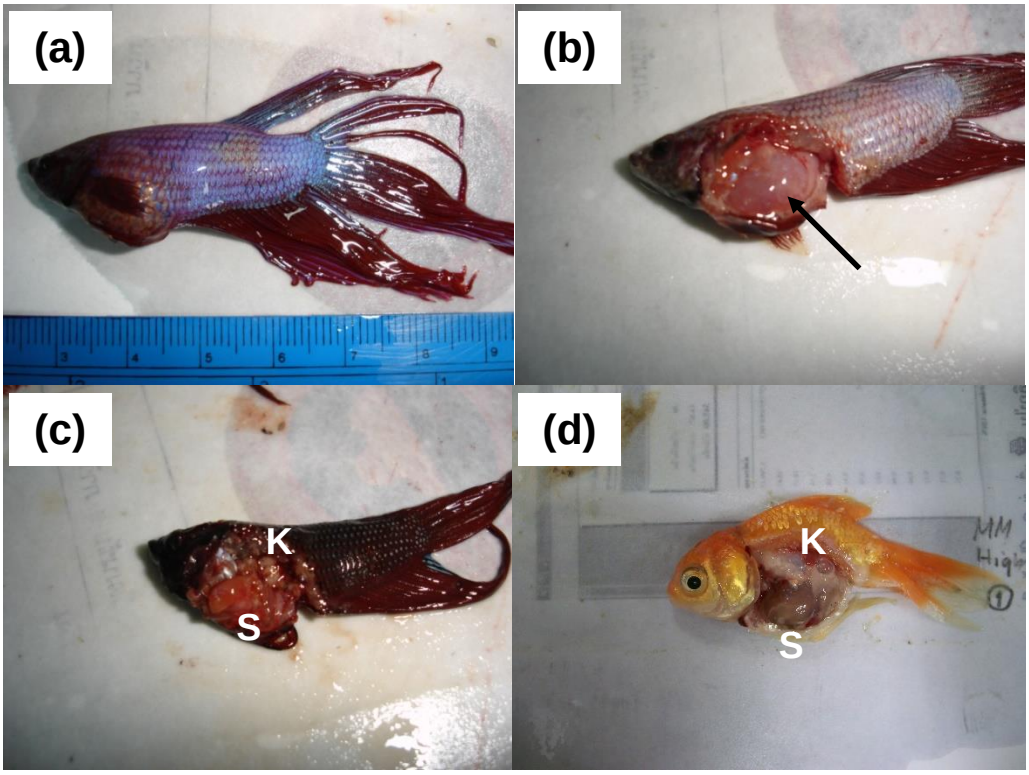

Figure 1 Gross lesions of Siamese fighting fish (a, b, c) and goldfish (d). (a) Abdominal distension. (b) Ascites (black arrow). (c, d) Hepatomegaly and ascites, kidney enlargement (K), splenomegaly (S).

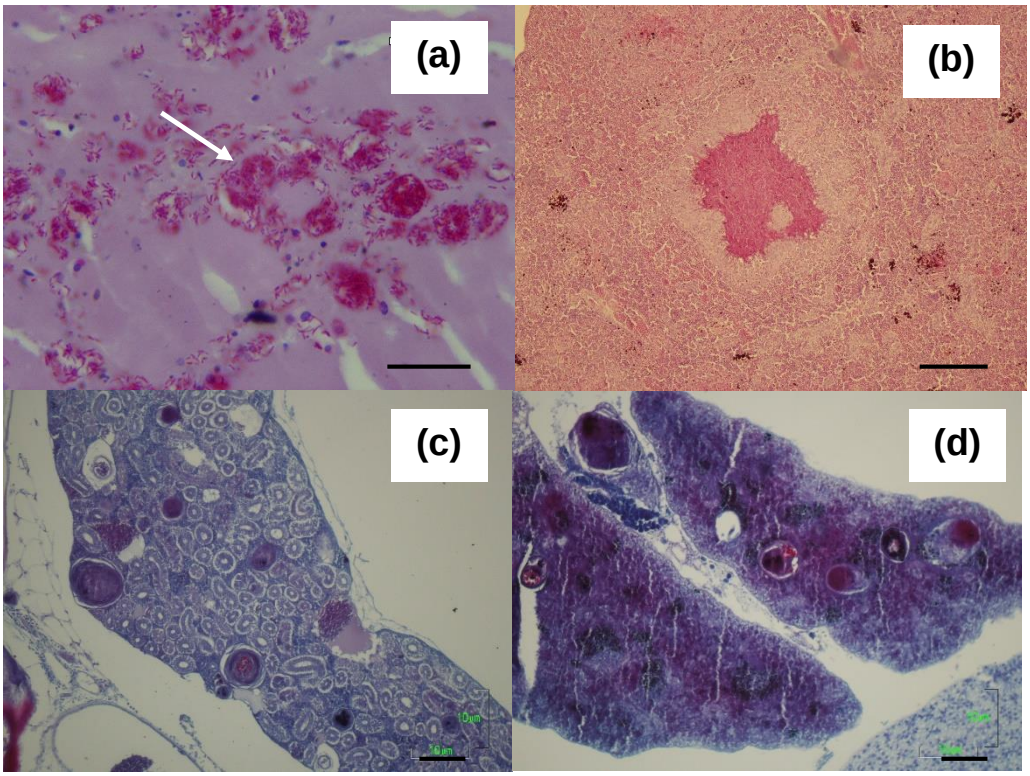

Figure 2 (a) Acid-fast bacteria in examined tissue (white arrow). Ziehl-Neelsen staining. Bar = 20 µm. (b) *Mycobacterium* was observed in the center, and the large proliferating fibroblast and multiple layers of epitheloid cells were found in the periphery. Ziehl-Neelsen staining. Bar = 30 µm. (c) Granulomatous nephritis. Ziehl-Neelsen staining. Bar = 10 µm. (d) Granulomatous splenitis. Ziehl-Neelsen staining. Bar = 10 µm.

Table 2 Biological and biochemical characteristics of *Mycobacterium* spp., isolated from Siamese fighting fish

Isolate	Gram stain	Acid-fast stain	Motility	Colony type	Pigmentation	Growth rate at 25C	Degradation of PAS	Nitrate reduction	Tween hydrolysis, 5 days	Arylsulfatase, 3 days	Arylsulfatase, 14 days	Urease	Picric acid
M. marinum	+[a]	+	N[b]	S/ SR[c]	P[d]	S[e]	-[f]	-	+	-	+	+	-
M.fortuitum subsp.acetamidolyticum	+	+	N	Sf/Rf	N	R	-	+	V[g]	+	ND[h]	+	-
M. fortuitum subsp.fortuitum	+	+	N	Sf/Rf	N	R	+	+	V	+	ND	+	+
M. fortuitum subsp.peregrinum	+	+	N	Sf/Rf	N	R	+	+	V	+	ND	+	+
M. chelonae subsp.abscessus	+	+	N	S/R	N	R	+	-	V	+	ND	+	+
M. chelonae subsp.chelonae	+	+	N	S/R	N	R	+	-	V	+	ND	+	-
KKVB0901-KKVB0915	+	+	N	S	P	S	-	-	+	-	+	+	-
KKVB0916-KKVB0925	+	+	N	S	P	S	-	-	+	-	+	+	-
KKVB0926-KKVB0935	+	+	N	R	N	R	+	+	+	+	+	+	+
KKVB0936-KKVB0945	+	+	N	S	P	S	-	-	+	-	+	+	-
KKVB0946-KKVB0955	+	+	N	R	N	R	+	+	+	+	+	+	+
KKVB0956-KKVB0959	+	+	N	S	P	S	-	-	+	-	+	+	-
KKVB0960-KKVB0963	+	+	N	R	N	R	+	+	+	+	+	+	+

* Modified from Herbert and Robert (1985), Sneath *et al.,* (1986) and Barrow and Feltham (1993). a: Positive, b: Non-motility, c: S, smooth; SR, intermediate in roughness; R, roughness; f, filamentous, d: P, photochromogenic; N, nonphotochromogenic e: S, slow; M, moderate; R, rapid, f: negative, g: V, variable, h: ND, not determined, i: d, 11 – 89% of strains are positive

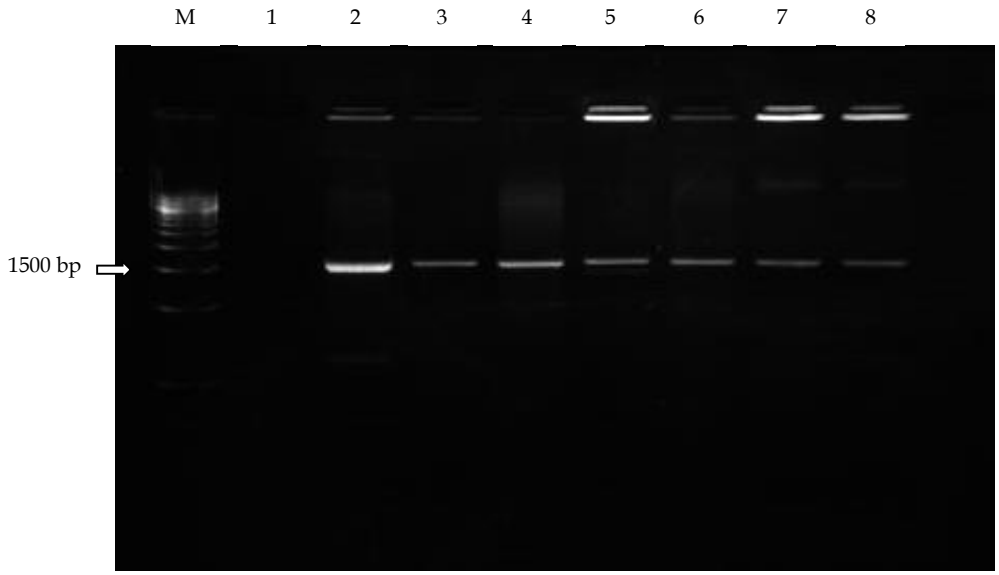

Figure 3 The results of PCR amplification of *Mycobacterium* spp. isolated from fishes by electrophoresis. Lane M, marker; lane 1, negative control; lane 2, *Mycobacterium marinum* NJB0419; lane 3, *Mycobacterium chelonae* NJB0414; lane 4, *Mycobacterium fortuitum* NJB0505; lane 5, KKVB0901; lane 6, KKVB0902; lane 7, KKVB0926; lane 7, KKVB0927.

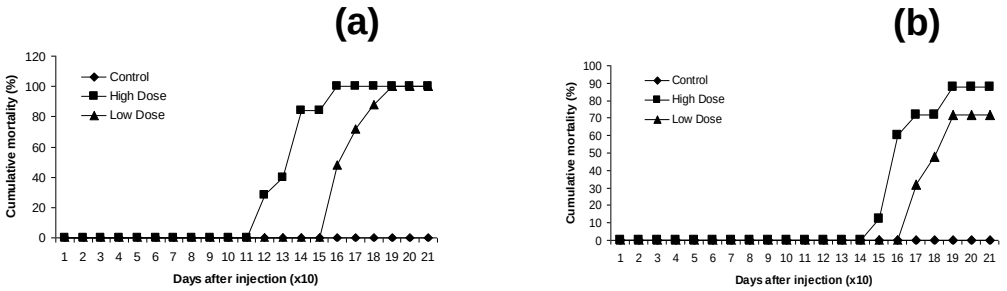

Figure 4 The cumulative mortality of goldfish intramuscularly injected with (a) *Mycobacterium marinum* KKVB0901 and (b) *Mycobacterium fortuitum* KKVB0926 suspension of high and low dose.

The clinical signs and histological features of the experimentally infected goldfish closely resembled those observed in Siamese fighting fish naturally infected with the bacterium. The goldfish had retarded growth, cutaneous ulceration at the site of injection and abdominal distension. The necropsy findings were hepatomegaly, splenomegaly, kidney enlargement with white nodules and ascites (Fig 1d). Acid-fast bacilli stained with Ziehl-Neelsen were found in the granulomas and parenchyma of the examined tissues. Distinct lesions were disseminated granulomatous inflammation of kidney (Fig 2c), spleen (Fig 2d), liver, pancreas, muscle fiber and vertebrae. Most granulomas were also soft tubercle-type.

Discussion

The isolates were identified as *M. marinum and M. fortuitum* on the basis of morphological and biochemical characteristics as in previously reported papers (Herbert *et al*., 1985; Sneath *et al*., 1986; Barrow and Feltham, 1993) and of the analysis of a partial 16S rRNA gene sequence. The prevalence of *Mycobacterium* spp., in this study was 25.7% in clinically healthy fish but was 100.0% in the moribund fish that closely relates with a previous study (Pungkachonboon *et al*., 1992). However, the prevalence of mycobacterium infections in ornamental fish or fresh water fish may vary from as low as 1.7% in one study (Mrlik *et al*., 2012) to as high as 41.7% in another study (Slany *et al*., 2014) and is dependent on the methods of bacterial detection (Slany *et al*., 2014). The results of this study indicated that clinically healthy Siamese fighting fish from cultured farms and ornamental fish markets in Thailand were reservoir for *M. marinum* and *M. fortuitum* with lower prevalence and more difficult to detect compared with the moribund fish. Therefore, concern should be raised for people at risk especially the sellers or the buyers to avoid mycobacterial infections from clinically healthy Siamese fighting fish.

Results of this study also showed that there was high morbidity of mycobacteriosis in Siamese fighting fish in Thailand. Although most naturally infected Siamese fighting fish presented with subclinical signs, clinical signs and histopathological findings of the moribund fish closely resembled those observed in experimentally infected goldfish. The clinical signs of the experimentally infected goldfish were lethargy,

anorexia, emaciation, abdominal distension and ascites. Histopathological findings were disseminated granulomatous inflammations of multi-organs which were found similarly in other species of affected fish (Majeed *et al*., 1981; Shamsudin *et al*., 1990; Gómez, 1998; Brocklebank *et al*., 2003; Overton *et al*., 2003; Weerakhun *et al*., 2007). Moreover, all granulomas were classified as a soft tubercle-type that could be explained as the bacteria being weakly harmful pathogens, slow growing and slow progression, or on early stage of the diseases (Hatai *et al*., 1993; Puttinaowarat *et al*., 2002; Brocklebank *et al*., 2003; Overton *et al*., 2003; Weerakhun *et al*., 2008). Histopathological findings at the periphery of granulomas were the multiple layers of epitheloid cells and proliferating fibroblasts. Numerous acid fast bacilli could be found in the examined organs (Brocklebank *et al*., 2003; Weerakhun *et al*., 2008).

Results from the pathogenicity test showed that *Mycobacterium marinum* KKVB0901 and *M. fortuitum* KKVB0926 isolated from Siamese fighting fish were effective pathogens in goldfish. The cumulative mortalities of the goldfish injected with both bacteria were depended on the dose (Weerakhun *et al*., 2008). Moreover, *M. marinum* KKVB0901 accidentally infected one of the authors on his finger by unintentional injection of the bacterium. The affected site showed cutaneous ulceration with pustules after 4 weeks of injection (Fig 5). He was treated with clarithromycin for 2 months and recovered. This iatrogenic case confirmed the pathogenicity in human that *M. marinum* can cause skin lesions in human (Johnston and Izumi, 1987; Edelstein, 1994; Ramakrishnan, 1997; Ryan and Bryant, 1997; Lim *et al*., 2000; Weitzul *et al*., 2000). In severe cases, other lesions may be found such as chronic breast abscess, rhinitis, arthritis, tenosynovitis and bone lysis (Harth *et al*., 1994; Barton *et al*., 1997; Shih *et al*., 1997; Saadatmand *et al*., 1999; Van Seymortier *et al*., 2004; Lam *et al*., 2006).

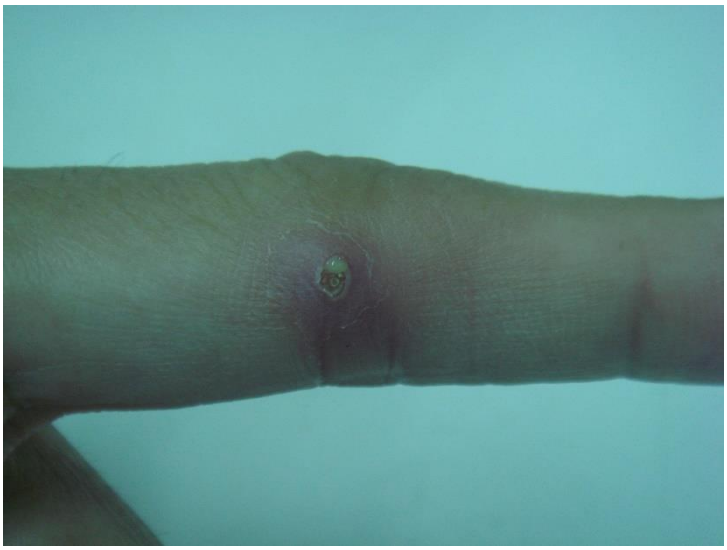

Figure 5 Accidental infection in one of the authors with *Mycobacterium marinum* KKVB0901. The skin inflammation was found in the second week after infection and then the lesion was progressed week by week.

In conclusion, this study showed high morbidity of *Mycobacterium* infections in Siamese fighting fish. Two species, *M. marinum* and *M. fortuitum*, were isolated and these can be potent pathogens in goldfish, confirmed by pathogenicity test. The clinical signs and histological findings of moribund Siamese fighting fish were similar to those of experimentally infected goldfish. Moreover, the accidental infection with *M. marinum* in a human strengthened its effective, zoonotic pathogen.

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