# Studies on nontuberculous mycobacterium ; *Mycobacterium* sp. pathogenic for filefish

Summary of Doctoral Thesis

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Mycobacterial species except for *Mycobacterium tuberculosis* complex (*M. africanum, M. bovis, M. canettii, M. caprae, M. microti, M. orygis, M. pinnipedii*) and *M. leprae* crade has been called as “nontuberculous mycobacteria (NTM)”.

According to the Runyon classification, NTM are classified into four groups by their growth rate (SGM: slowly glowing mycobacteria and RGM: rapidly growing mycobacteria) and their photochromogenicity.

Infectious diseases caused by NTM have been reported in more than 165 fish species in saltwater, brackish water and freshwater, regardless of wild, ornamental or aquaculture species. *M. marinum, M. chelonae, M. fortuitum, M. abscessus, M. chesapeaki, M. shottsii, M. pseudoshottsii* and *M. salmoniphilum* are the most commonly identified NTM species as fish pathogen.

In 2009, high levels of mortality were observed in thread-sail filefish, *Stephanolepis cirrhifer*, at a fish farm in Ehime prefecture, Japan. Acid-fast, non-photochromogenic rapid-growing NTM were isolated from the dead fish. DNA-DNA hybridization tests showed that the representative strain, NJB0901, was closely related to *M. chelonae*.

From 2009 to 2013, similar infectious disease cases have been reported in both farmed and wild thread-sail filefish, and farmed black scraper (*Thamnaconus modestus*) populations in several areas of Japan. Black scraper is a closely related species to thread-sail filefish. Some NTM strains were isolated from the infected fish of these cases.

In Chapter 2, twenty-six NTM isolates from the infected file-fishes (thread-sail filefish and black scraper) were characterized using biological and biochemical analyses. In addition, susceptibility tests for antibiotics were also performed with the strains.

These isolates showed identical biological and biochemical characteristics. Growth

occurred at 15–35°C, with the optimum temperature being 30°C. Most colonies appeared rough and white colored without pigmentation after incubation at 30°C for 5 days .

The filefish isolates and *M. chelonae* JCM 6388$^{T}$ showed positive results for catalase activity at 68°C and negative for growth on medium containing picric acid, while *M. salmoniphilum* ATCC13758$^{T}$ showed negative and positive results for the two tests, respectively.

In contrast, the filefish isolates and *M. salmoniphilum* ATCC13758$^{T}$ showed very weak growth on the media containing 5% NaCl, while *M. chelonae* JCM6388$^{T}$ showed apparent growth on it. From the results, it was indicated that these biological and biochemical tests could be useful to distinguish NTM species among the file-fish strains, *M. chelonae* and *M. salmoniphilum*.

The filefish isolates showed relatively low MIC values with erythromycin and were susceptible to clarithromycin, doxycycline, and ciprofloxacin.

In Chapter 3, transmission trials were performed to evaluate the invasion route of NTM into thread-sail filefish, and pathogenicity of the strain isolated from thread-sail filefish against black scraper.

Transmission trials were performed by immersion, peroral administration and intraperitoneally injection, however, only intraperitoneally injection could reproduce the typical features of file fish NTM infection. The results suggested that some other factors assisting NTM infection might involve in the spontaneous infection.

In the pathogenicity test for black scraper, the cumulative mortality of the experimental group exceeded 50% at 4 days after inoculation and reached 100% at the end of the experiment. This showed the strain isolated from filefish was also pathogenic to black scraper. The dead fish showed similar histopathological features to those in the thread-

sail filefish characterized by pyogranulomatous lesions on the surface of serosae of alimentary tract and mesentery.

The most common and prominent pathological features of piscine NTM infection are enlargement of the spleen and kidney associated with greyish-white nodules consisted of typical epithelioid cellular granulomas with NTM on these organs, therefore, these organs have been used for isolation of the pathogens. In contrast, such pathological features could not be detected among the diseased filefish in the present study. From the results, it was likely that spleen and kidney could not be suitable for isolation of the pathogens in filefish NTM infections.

In Chapter 4, multi locus sequence typing and molecular phylogenetic analysis, 65-kDa heat shock protein (*hsp*65) PCR restriction enzyme digestion assay (PRA) analysis and pulsed field gel electrophoresis (PFGE) were performed.

PCR and sequencing analyses were performed targeting the 16S rRNA gene, RNA polymerase β-subunit (*rpo*B) gene, 65-kDa heat shock protein (*hsp*65) gene, recombinase A (*rec*A) gene, and superoxide dismutase A (*sod*A) gene.

The partial sequences of these five genes showed 100% similarity among the tested filefish strains. Representative sequences from designated filefish type strain NJB0901 have been deposited in the GenBank database (16S rRNA: AB971866; *rpoB*: LC008146; *hsp65*: LC008145; *recA*: LC008147; and *sodA*: LC008148).

In the phylogenetic tree based on the 16S rRNA gene sequences, NJB0901 was located in the *M. chelonae-M. abscessus* group. As for the other four genes, NJB0901 was classified into the same cluster with *M. chelonae*.

In the tree generated from the concatenated data of 16S rRNA, *hsp65*, *rpoB*, *recA*, and *sodA* genes sequences, NJB0901 was distinctly separated from *M. salmoniphilum* and *M.*

*chelonae*, with high bootstrap values among them.

PRA pattern analyses of hsp65 were performed using NJB0901, *M. chelonae* JCM6388$^T$, and *M. salmoniphilum* ATCC13758$^T$. Aliquots of the resulting amplicons of hsp65 gene were then digested with restriction enzymes *Bst*EII or *Hae*III. Digested products were electrophoresed and observed by UV transilluminator.

Additionally, the *Bst*EII and *Hae*III restriction sites within the *hsp65* sequences of tested strains were investigated virtually using GENETYX ver 11.0 to determine the sizes of the expected fragments. These fragment sizes were compared with those of other mycobacterial species in the PRASITE database.

All tested strains showed an identical pattern followed by digestion with *Bst*EII. These findings were supported by the results of the GENETYX program. However, the NJB0901 was predicted to produce bands of 220, 54, and 58 bp following *Hae*III digestion, while *M. chelonae* and *M. salmoniphilum* were expected to produce bands of 197, 60, 54, and 58 bp in the same reaction, respectively. Therefore, *Hae*III digestion could be used to distinguish NJB0901 from the control type strains examined in this study.

Pulsed field gel electrophoresis analysis was performed with six filefish isolates originated from different fish species, the areas of sea around Japan and the isolation periods. The completely same PFGE patterns were observed in all tested strains treated with two different restriction enzymes (*Xba* Ⅰ、*Ase* Ⅰ). This results showed all the strains isolated from different conditions have same genotypic character, and it was also suggested that filefish NTM species has already colonize in considerably wide area of sea around south-western Japan.

In Chapter 5, protein profiling and lipid profiling were performed by MALDI-TOF MS. Protein profiling using MALDI Biotyper 3.1 (Bruker Daltonics, Inc.) was

performed with filefish strain NJB0901, *M. chelonae* JCM6388$^{T}$, and *M. salmoniphilum* ATCC13758$^{T}$.

Mass spectra were acquired by autoflex speed (Bruker Daltonics, Inc.) and the homology of NJB0901 with two type strains was evaluated by the Biotyper score values.

The score values for NJB0901 against two type strains, *M.chelonae* JCM6388$^{T}$ and *M.salmoniphilum* ATCC13758$^{T}$ were 1.893 and 1.301 respectively. According to the manufacture's instruction, these results suggested that NJB0901 have unique protein profile.

MALDI-TOF MS analysis of total lipids was performed using filefish strain NJB0901$^{T}$, *M. chelonae* JCM6388$^{T}$, and *M. salmoniphilum* ATCC13758$^{T}$. Total lipids were extracted from the bacteria cultured on Middlebrook 7H11 agar with/without Tween80.

The mass spectrum patterns of NJB0901 and *M.chelonae* showed distinctive 44 amu-interval clusters, which only appeared when cultured with Tween 80. In addition, these two strains did not share the mass range. These results suggested that NJB0901 possessed a unique metabolic mechanism for Tween 80.

In Chapter 6, PCR primers set was designed for specific detection of *Mycobacterium* sp. isolated from filefish, and evaluated the availability for rapid diagnosis. The primers set was designed by using draft genome sequences acquired by next-generation sequencer. The designed specific primers, M ste-F and M ste-R, were specifically reacted with *Mycobacterium* sp. isolated from filefish and did not react with other NTM type strains. The detection limit was 1pg/uL of genomic DNA extracted from pure cultured NJB0901. The result indicated high availability of the PCR primers set for rapid detection of filefish NTM pathogen.

In addition, detection tests for bacterial cells in the kidney and spleen of black scraper

experimentally infected with filefish NTM were performed. PCR primers set could detect $10^3$ CFU of mycobacterial cells as the lowest level. From the results, the author concluded that the detecting methods would have room for improvement in efficiency of DNA extraction or choosing the appropriate specimens.