Studies on transmission cycles of *Mycobacterium marinum* in closed-rearing environment of aquaria in Japan

Summary of Doctoral Thesis

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Mycobacterium marinum is a nontuberculous mycobacterium (NTM). NTM includes a lot of mycobacterial species with the exception of *Mycobacterium tuberculosis* complex and *Mycobacterium leprae. M. marinum* infection mainly causes multifocal granulomatous nodules on the skin of the fingers, hands and arms in humans, which is especially common among aquarium keepers or people swimming in natural. In addition to humans, it has been reported that *M. marinum* causes infectious diseases to the other mammals, fish, amphibians, reptiles, birds, and arthropods. Furthermore, *M. marinum* is ubiquitous, especially distributed in aquatic environments. Up to the present, the pathogen has been also isolated from various aquatic creatures such as mollusks, echinoderms, and amoebae, and from various environmental components such as sand, water, biofilm, algae, and plants in natural waters (fresh, estuarine, and sea waters). Abundant previous reports have also described that this bacterial species was isolated from rearing environments (tanks at individual homes, aquaria, and aqua-culture farms). These evidences lead to hypothesize that a wide variety of animals and environmental factors could be involved in transmission cycles of *M. marinum*. However, that has not been proven sufficiently yet.

The present study aims to confirm the hypothesis about transmission of *M. marinum* in closed-rearing environment using molecular epidemiological methods. We diagnosed *M. marinum* infection and performed isolation of this pathogen from various animals and environmental components in two aquaria (aquarium A and B) in Japan (Chapter 2 & 3). Furthermore, we conducted phylogenetic analyses in obtained isolates (Chapter 4).

Chapter 2：a case of *M. marinum* infection –aquarium A–

In this chapter, to diagnose *M. marinum* infection in flyingfish reared in a tank (tank A) in aquarium A, where continuous mortality occurred in 2017–2020, we conducted necropsy, histopathological examination, isolation of the pathogen, and molecular biological examination in collected diseased fish (n = 53; sharphead flyingfish *Hirundichthys oxycephalus*). By necropsy, multifocal whitish micronodules on the internal organs were revealed. In the histopathological examination, granulomas associated with acid-fast bacilli were observed in 48 out of 50 individuals examined. In

bacterial isolation, mycobacterial isolates were obtained from 47 out of 50 individuals examined, and most of the isolates were classified into Runyon group I. In the molecular biological examination, the concatenated tree revealed that selected isolates classified into Runyon group I and tissue-extracted samples formed a cluster with *M. marinum*. Additionally, the isolates were tested for the presence of insertion sequences IS*2404* and IS*2606*, and the pattern (IS*2404* positive/IS*2606* negative) of the selected isolates matched with the pattern of some *M. marinum* strains. Therefore, isolates belonging Runyon group I, which occupied a majority of isolates, were identified as *M. marinum*. Based on the results, we concluded that the fish examined were diangnosed with mycobacteriosis mainly associated with *M. marinum*. In addition, mycobacteriosis with a dematiaceous fungus (*Exophiala aquamarina*) was observed in 13 out of 53 individuals examined.

After diagnosis of *M. marinum* infection in flyingfish in tank A, to investigate infiltration conditions and transmission cycles of the pathogen in aquarium A, we performed isolation from wild and reared fish (n = 50; five species of flyingfish and slender wrasse *Suezichthys gracilis*) and environmental components (n = 41; biofilm, rearing water, pouring water, bottom sand, and food) in 13 tanks (tank A–K) in the aquarium. The bacterial strains belonging *M. marinum* were isolated from four species of flyingfish and filter sand in tanks rearing flyingfish (tank A, B). *M. marinum* was also obtained from the wrasse and bottom sand in the tank exhibiting eelgrasses *Zostera marina* bed (tank G). Thus, it was speculated that factors including fish and environmental components such as bottom sand or filter sand could be involved in transmission cycles of *M. marinum* in the aquarium, and the pathogen could infiltrate into at least two tanks.

Chapter 3：a case of *M. marinum* infection –aquarium B–

In this chapter, to diagnose *M. marinum* infection in fish reared in a tank (tank M) in aquarium B, where continuous mortality occurred in 2018–2020, we conducted necropsy, histopathological examination, isolation of the pathogen, and molecular biological examination in collected dead fish (n = 4; pygmy filefish *Rudarius ercodes*, Japanese sea catfish *Plotosus japonicus*, redfin velvetfish *Hypodytes rubripinnis*). In the

histopathological examination, the granulomas associated with acid-fast bacilli were observed in the one pygmy filefish. By isolation of the pathogen, mycobacterial isolates were obtained from the filefish and classified into Runyon group I. In the molecular biological examination, the concatenated tree revealed that selected isolates classified into Runyon group I and tissue-extracted samples formed a cluster with *M. marinum*. In addition, the isolates were tested for the presence of insertion sequences IS*2404* and IS*2606*, and the pattern (IS*2404* positive/IS*2606* negative) of the isolates matched with the pattern of some *M. marinum* strains. Therefore, we concluded that at least the one filefish examined could be diagnosed as mycobacteriosis associated with *M. marinum*.

After diagnosis of *M. marinum* infection in the fish in tank M, to investigate infiltration conditions and transmission cycles of the pathogen in aquarium B, we performed isolation from wild and reared animals (n = 66; fish, n = 20; invertebrates) and environmental components (n = 152) in the seashore where eelgrasses were collected and in 13 tanks (tank L–K) in the aquarium. *M. marinum* was isolated from eight species of fish, nine species of invertebrates (ragworm, mollusks, echinoderm, shrimp, hermit crab, and sea cucumber), and environmental components (rearing water, bottom sand, filter sand, biofilm, algae, and eelgrass) in the tank exhibiting eelgrasses bed (tank M). Thus, it was likely that various factors including fish, invertebrates, and environmental components could be involved in transmission cycles of *M. marinum* in tank M in the aquarium.

Chapter 4： Investigation of transmission cycles by molecular epidemiological analyses

In this chapter, in order to confirm transmission cycles of *M. marinum* in aquarium A and B, we conducted molecular epidemiological analysis (core single nucleotide polymorphism (SNP) analysis) in isolates from Chapter 2 and 3. Additionally, to evaluate usefulness of variable number of tandem repeats (VNTR) analysis as a rapid method, a comparison of VNTR and core SNP analyses was also conducted.

Core SNP analysis was performed with 25 strains including isolates originated from aquarium A and B, isolates obtained in the other cases and stored in our laboratory, and type strains. The analysis classified isolates from aquarium A and B into four clusters

(genotype i–iv). In aquarium A, isolates from the wrasse and bottom sand in the tank exhibiting eelgrasses bed (tank G) were classified into genotype i. In addition, isolates from flyingfish and filter sand in the tanks rearing flyingfish (tank A, B) were classified into genotype iii. Thus, it was suggested that fish and environmental components could be involved in transmission cycles of *M. marinum* in aquarium A. It was also suggested that transmission cycles of the pathogen in the tank with flyingfish and the tank with eelgrasses bed may be independent. For aquarium B, most of isolates from fish, invertebrates, and environmental components in the tank exhibiting eelgrasses bed (tank M) were classified into Genotype iv, and the only two strains from the mullet classified into genotype ii. Hence, it was confirmed that these various factors such as fish, invertebrates, and environmental components could be involved in transmission cycles of *M. marinum* in aquarium B. Furthermore, it was likely that genotype iv could fill the ecological niche of *M. marinum* in the tank.

VNTR analysis for 11-loci of *M. marinum* gene was performed with 46 strains (including the strains used for core SNP analysis). The analysis divided isolates from aquarium A and B into 3 genotypes as follows. The strains classified into genotype i belonged to genotype 1, and the two strains classified into genotype ii belonged to genotype 2. The strains classified into genotype iii or iv belonged to genotype 3. Therefore, we conducted the second VNTR analysis with additional 6 VNTR loci which have been previously reported and newly generated 14 loci (V1, V2, V3, V4, V5, V6, V7, V-2-7, V-2-12, V-2-31, V-2-36, V-2-39, V-2-41) to improve the resolving ability of VNTR analysis. However, the second analysis was not yet able to discriminate those isolates belonging genotype 3 into two subgroups of aquarium A-starins and aquarium B-strains.

In this study, to confirm transmission cycles of *M. marinum*, we conducted the phylogenetic analysis in the *M. marinum* stains from two aquaria in Japan. In addition, to evaluate usefulness of VNTR analysis, a comparison of VNTR and core SNP analyses was also conducted. This molecular-based study epidemiologically confirmed that various factors including fish, invertebrates and environmental components could be involved in transmission cycles of *M. marinum* in closed-rearing environments.

Furthermore, it was conducted that further investigation of VNTR locus sets is needed to utilize VNTR analysis.