Mycobacterium marinum Infection in Cultured Yellowtail Seriola quinqueradiata in Japan

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ABSTRACT—Recently, *Mycobacterium* infection has been observed in cultured yellowtail *Seriola quinqueradiata* in Japan but not studied in detail. Diseased fish were lethargic, anorexic and emaciated, and showed hemorrhagic cutaneous ulceration and ascites. The necropsy and histopathological features showed that disseminated necrosis and numerous white nodules were found in the kidney, spleen, liver and heart. Numerous acid-fast bacteria were detected in the above tissues and granulomas. Myositis, hepatitis, splenitis and nephritis due to granulomas and gill inflammation were histologically observed. Almost all granulomas were classified into soft tuberculosis. All bacteria isolated from the diseased fish were Gram-positive, acid-fast, rod and non-motile. As a result, they were classified into the genus *Mycobacterium*. The isolates were identified as *Mycobacterium marinum* on the basis of biological and biochemical characteristics and the analysis of a partial 16S rRNA gene sequence. An experimental infection test showed that a representative isolate had pathogenicity to yellowtail with disease signs similar to those of naturally affected fish. This is the first report on *M. marinum* infection in cultured yellowtail.

Key words: *Mycobacterium marinum*, *Seriola quinqueradiata*, yellowtail, fish disease

*Mycobacterium* infection has been found in freshwater and marine fish over 150 species (Chinabut, 1999). At the present, *Mycobacterium marinum*, *M. fortuitum* and *M. chelonae* (Sneath et al., 1986; Frerichs, 1993) in the genus *Mycobacterium* are known as fish pathogens (Chen et al., 1997; Heckert et al., 2001) and *M. marinum* sometimes causes skin disease in human (Lim et al., 2000). In addition, Heckert et al. (2001), Rhodes et al. (2003) and Rhodes et al. (2005) reported new species, *M. chesapeake*, *M. shottii*, and *M. pseudoshottii*, respectively, from striped bass *Morone saxatilis* in Chesapeake Bay. Levi et al. (2003) also reported a new species, *M. montefiorense* from captive moray eels *Gymnothorax funebris* and *G. moringa*. Kusuda et al. (1987) reported *Mycobacterium* infection in yellowtail, which occurred in September 1985 to February 1986 caused by a photochromogenic mycobacterium. The pathogen differed from previously reported species when compared with some biological characteristics and optimum temperature for growth. They reported that it as *Mycobacterium* sp., but they did not mention the species in the paper.

In October 2004, diseased yellowtail with *Mycobacterium* infection were found in southern Japan. It was similar in fish size, season, disease signs, and bacterial characteristics compared with *Mycobacterium* sp. reported by Kusuda et al. (1987). We isolated some strains from diseased fish, and studied on histopathological features, biological and biochemical characteristics, pathogenicity to yellowtail, and phylogeny.

**Materials and Methods**

**Isolation**

Moribund 12 yellowtail, six fish (1,626–4,360 g, 46–61.5 cm) examined in October 2004 and other six fish (703.5–2,860 g, 41–49 cm) in February 2005, were collected at fish farms in Kagoshima Prefecture.

Bacterial isolation was attempted from the kidney,
spleen, liver and gills of each fish. The small pieces of each tissue were homogenized with 4% NaOH for 10 min and each solution was inoculated on 1% Ogawa medium (Nissui seiyaku). Middlebrook 7H10 agar with OADC (Becton Dickinson) and BHI agar (Becton Dickinson) by a loop. In addition, each tissue was also inoculated on BHI agar without treatment with 4% NaOH for detection of other bacteria. They were incubated at 25°C for 2 months and then visible colonies were purified.

**Histopathology**

The gills, spleen, kidney, heart and liver from the moribund fish were fixed in 10% phosphate-buffered formalin solution, and later the gills was decalcificated with 10% EDTA solution. They 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.

**Identification**

Characteristics: Eight out of 12 strains isolated from 12 moribund yellowtails were selected at random and were used for identification (Table 1). 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). The morphologies of 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 et al. (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. They were also tested for detection of other bacteria. They were incubated at 30°C for 2 months and then visible colonies were purified at 5% NaCl were examined as described by Herbert et al. (1985) and Barrow and Feltham (1993).

**Pathogenicity test**

Ninety yellowtail (50–100 g in body weight and 17–20 cm in total length) were used for experimental infection. They were fed pellets everyday. They were divided into three groups and each group had 30 fish. *Mycobacterium* sp. NJB 0419 was adjusted to 2.2 × 10^6 and 2.2 × 10^4 CFU/mL in 0.85% NaCl and intramuscularly injected with the bacterial suspension at a dose of 0.2 mL per 100 g body weight. The control fish were injected with 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 experiment, 30 days, the survival fish were killed and re-isolations of bacteria were conducted.

**Results**

**Disease signs**

The fish naturally infected with the mycobacteria showed lethargic, anorexia, emaciation and abdominal distension with ascites. The ascites was characterized...
Fig. 1. Skin ulceration and subcutaneous granulomas. Bar = 3.5 cm.
Fig. 2. Eye exophthalmos and eye collapsed. Bar = 1 cm.
Fig. 3. White nodules found on gills (arrow). Bar = 1 cm.
Fig. 4. Swelling of spleen (S) and kidney (K) with numerous white nodules. Bar = 2 cm.
Fig. 5. Acid-fast bacteria in heart (arrows). Ziehl-Neelsen stain. Bar = 20 μm.
Fig. 6. Granulomatous splenitis: soft tubercle type. H&E stain. Bar = 30 μm.
Fig. 7. Granulomatous nephritis: hard tubercle type. Ziehl-Neelsen stain. Bar = 20 μm.
Fig. 8. A granuloma found in gills. Ziehl-Neelsen stain. Bar = 20 μm.
by pale yellow fluid. Hemorrhages were observed on skin, mouth, lower jaw and operculum. In severe cases, hemorrhagic cutaneous ulceration and subcutaneous erythematous granulomas were observed (Fig. 1). Eye exophthalmos, corneal ulceration and collapsed (Fig. 2) were observed in some cases. Numerous white nodules were generally found in several internal organs including the kidney, spleen, liver, heart and gills (Fig. 3), especially the spleen and kidney. Hepatomegaly with focally disseminated necrosis and jaundice, splenomegaly and kidney enlargement were observed (Fig. 4).

**Histopathological features**

Numerous acid-fast bacilli stained with Ziehl-Neelsen were found in granulomas and parenchyma of examined tissues (Fig. 5). Disseminated granulomatous myositis, granulomatous hepatitis, granulomatous splenitis (Fig. 6), interstitial nephritis (Fig. 7) and gill inflammation (Fig. 8) were focally observed. Spleen, kidney and liver were histopathologically 100% positive for the presence of granuloma and heart and gills were 75% and 29%, respectively. Spleen showed the highest damaged due to the infection in the examined tissues.

**Identification**

All isolates were Gram-positive, acid-fast, non-motile and photochromogenic. They grew at 15 to 37°C with an optimum temperature of 25°C on Middlebrook 7H10 agar. They did not grow on 5% NaCl Middlebrook 7H10 agar. They were negative for reduction of nitrate and degradation of PAS. They resisted inhibition by picric acid, and were positive for reduction of arylsulfatase (at day 10), urease, Tween 80 hydrolysis (at day 5), and semi-quantitative catalase (Table 2). In addition, they were negative for acid formation from 19 carbohydrates (arabinose, mannitol, rhamnose, xylose, ducitol, maltose, levulose, glucose, mannose, inositol, glycerol, lactose, raffinose, sorbose, trehalose, arbutin, adonitol, galactose and cellubiose). They were also negative for carbon source utilization of 11 carbon sources (tartarate, malonate, lactate, fumarate, pyruvate, succinate, oxalate, acetate, benzoate, propionate and citrate), and for protein decomposition of tyrosine, xanthine, hypoxanthine and casein.

Approximately 1,400 bp length of a partial 16S rRNA gene about four isolates were analysed and the sequences were completely matched (100%) each other. The phylogenic tree with the isolate NJB 0419 and the related *Mycobacterium* spp. were shown in Fig. 9. The NJB 0419 belonged to a cluster with *M. pseudoshottsi* and *M. marinum*, and had no difference with *M. pseudoshottsi* (AY570988) and 1 bp difference with *M. marinum* (AF456247) in the partial 16S rRNA gene sequence.

**Pathogenicity test**

Cumulative mortality of the fish injected with isolate NJB 0419 (Fig. 10) was dependent on the dose. The

### Table 2. Biological and biochemical characteristics of *Mycobacterium* sp. isolated from yellowtail

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<tr>
<th>Isolate</th>
<th>NJB 0418</th>
<th>NJB 0419</th>
<th>NJB 0420</th>
<th>NJB 0421</th>
<th>NJB 0422</th>
<th>NJB 0423</th>
<th>NJB 0501</th>
<th>NJB 0502</th>
<th>M. marinum*</th>
<th>M. chelonae*</th>
<th>M. fortuitum*</th>
<th>M. pseudoshottsi**</th>
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<td>Growth rate at 25°C</td>
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<td>Arylsulfatase (3 days)</td>
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<td>Arylsulfatase (10 days)</td>
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*Modified from Herbert and Robert (1985), Sneath et al. (1986) and Barrow and Feltham (1993), and **modified from Rhodes et al. (2005). *, positive; –, negative; +/-, variable up on subspecies; D, 11–89% positive; V, variable; ND, not determined; N, non-motile; S, smooth; SR, intermediate in roughness; R, rough; f, filamentous; P, photochromogenic; NP, nonphotochromogenic; SI, slow; M, moderate; Ra, rapid.
M. marinum infection in cultured yellowtail

Fish injected with higher dose (2.2 × 10⁶ CFU/mL) died at 100% within 23 days after inoculation, whereas the lower dose (2.2 × 10⁴ CFU/mL) was died at 100% on day 28.

The disease signs and histopathological features of the fish experimentally infected with Mycobacterium sp. NJB 0419 were closely resembled to those observed in fish naturally infected with the bacterium.

**Discussion**

All isolates in this study were identified as M. marinum according to general biological and biochemical characteristics in the previously reported papers (Herbert et al., 1985; Sneath et al., 1986; Barrow and Feltham, 1993). Molecular analysis revealed to be the same with the sequence of a partial 16S rRNA gene of four isolates randomly selected in this study. In the phylogenetic tree, Mycobacterium sp. NJB 0419 was most closely related to M. pseudoshottsii (AY570988), which is slowly growing photochromogenic species isolated from stripes bass, Morone saxatilis. The biological characteristics were little or no growth at 30°C, no growth at 37°C, negative for Tween 80 hydrolysis, arylsulfatase reduction (at day 3 and 14), and semiquantitative catalase (Rhodes et al., 2005). However, the four isolates from yellowtail including NJB 0419 showed clearly different characteristics from M. pseudoshottsii in the biological phenotypes and the findings suggest that the present isolates are not identified as M. pseudoshottsii. Recently, it was demonstrated that M. marinum has strain variation indicated by the difference of some nucleotides in 16S rRNA gene sequence (Ucko et al., 2002). This fact supports that our isolates identified as M. marinum, although they had 1 bp difference with M. marinum (AF456247) in the 16S rRNA gene sequence. Moreover, our isolates were different from those in the previous diseased yellowtail infection with Mycobacterium sp. (Kusuda et al., 1987), which was negative for Tween 80 hydrolysis characteristic (at day 5), and no growth at 37°C.

A pathogenicity test showed that Mycobacterium sp. NJB 0419 was highly pathogenic to yellowtail. Disease signs found in yellowtail were almost similar to the other fishes (Brocklebank et al., 2003; Overton et al., 2003). Numerous white nodules were generally observed in several internal organs as in other cases of Mycobacterium infection (Majeed et al., 1981; Shamsudin et al., 1990; Gomez, 1998; Brocklebank et al., 2003; Overton et al., 2003). Although, fish infected with Mycobacterium spp.
show subclinical sign or no external sign in the early stage of disease, it may be due to the slow growing of the pathogen and the slow progression of the disease (Hatai et al., 1993; Puttinaowarat et al., 2002; Brocklebank et al., 2003; Overton et al., 2003). Histopathological features revealed that the disease was a classical granuloma of the soft tubercule-type. The periphery of granulomas was the multiple layers of epitheloid cells and proliferating fibroblasts. Hard tubercles, which lack defined epitheloid layer and caseous necrosis, were observed (Hatai et al., 1993; Shamsudin et al., 1990). In examined tissue showed both exudative necrosis and granuloma necrosis, free numerous bacilli could find in examined organs (Brocklebank et al., 2003). *M. marinum* has been known as the causative agent of skin disease in human (Lim et al., 2000). Therefore, we are now planning to make a pathogenicity test of *M. marinum* NJB 0419 to mammals using mouse.

References


