養殖ブリから分離されたMycobacterium marinumの人為感染試験

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Artificial Infection of Mycobacterium marinum Isolated from Yellowtail Seriola quinqueradiata in Japan

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Abstract: Artificial infection was conducted to determine the pathogenicity of Mycobacterium marinum isolated from cultured yellowtail at a farm in Kagoshima Prefecture. Gross findings observed in the fish examined in this study were same with mycobacteriosis in cultured yellowtail naturally infected with Mycobacterium sp. In addition, histopathological features found in the fish of two and three weeks after inoculation were quite similar to those in the naturally infected fish. From the results, the bacterial strain used in this study was demonstrated to be a causative agent of the clinical case.

Key words: Mycobacterium marinum; Artificial infection; Yellowtail

Mycobacterium spp. cause Mycobacterium infection in over 150 species of freshwater and marine fishes (Chinabut 1999). Especially, Mycobacterium marinum has been recognized to be a highly pathogen in many fish species (Giavenni et al. 1980; Noga et al. 1989). In 2004, a massive mortality occurred in cultured yellowtail Seriola quinqueradiata at a farm in Kagoshima Prefecture, Japan. Weerakhun et al. (2007) have isolated acid-fast bacteria from the diseased fish, and identified them as M. marinum from their biochemical and molecular features. The clinical signs and histopathological features of the diseased fish were quite similar to those reported in the previous cases of fish mycobacteriosis (Mori et al. 1986; Kusuda et al. 1987; Hatai et al. 1993).

In this study, we would like to describe the details of histopathological changes found in yellowtails artificially injected with M. marinum NJM 0419, which was isolated from yellowtail naturally infected with the bacterium in Kagoshima Prefecture as mentioned above (Weerakhun et al., 2007).

Materials and Methods

Experimental fish
Thirty yellowtails were used for the artificial infection tests. These fish were obtained from a private fish farm in Kagoshima Prefecture and acclimated at Kagoshima Prefecture Fisheries Technology and Development Center. The fish were fed a commercial pellet. They were divided into two groups, experiment group and control and took 15 fish in each group.

Preparation for inocula
M. marinum NJM 0419 isolated from yellowtail on mycobacteriosis in 2004 was used in this study. The bacterium was incubated at 25°C for two months with Middlebrook 7H10 agar. Then, the colonies were suspended in sterile 0.85% NaCl, and adjusted to $2.2 \times 10^6$ CFU/ml for experiment group.

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Artificial infection

Before inoculation, fish were anesthetized with FA100 (Tanabe Co. Ltd., Tokyo). The bacterial suspension was inoculated 0.2 ml per 100 g body weight into the right dorsal trunk muscle under the dorsal fin with 25-gauge sterile needle. Control fish were inoculated 0.85% NaCl of 0.2 ml per 100 g body weight at the same site as the test fish. After inoculation, fish were kept in separated aquaria for three weeks. Water temperature in the aquaria was about 24°C during the course of experiments.

Five fish were collected from each experiment and control group at intervals of 1, 2 and 3 weeks after inoculation. After euthanization with overdose of FA100, the bacterium re-isolated from the kidney and/or spleen, and histopathological examination of the kidney, spleen, liver and gills were performed using the sampled fish.

Bacterial re-isolation and identification

The bacterium was re-isolated from the kidney and/or spleen by homogenizing these tissues with 4% NaOH for 10 min and inoculating on 1% Ogawa media (Nissui Co. Ltd, Tokyo), after observing gross findings from the moribund or freshly dead fish. The medium was incubated at 25°C for 2 months. Biological and biochemical characteristics of the isolates were examined and they were identified according to Manual of Clinical Microbiology (Herbert and Robert 1985) and Bergey’s Manual of Systematic Bacteriology (Sneath et al., 1986). The bacteriological features of the re-isolated strains were also compared with the original strain NJB 0419 (Weerakhun et al., 2007).

Histopathology

A necropsy was routinely performed. The kidney, spleen, liver and gills were then fixed in 10% phosphate buffered formalin solution, embedded in paraffin and sectioned at 4 to 5 μm. The sections were stained with hematoxylin and eosin (HE), Ziehl-Neelsen (ZN), Giemsa and Masson’s trichrome (MT).

Results

Clinical signs

Moribund fish showed lethargy, emaciation, and ulcerative dermatitis at the injected site. Grossly, these fish showed enlargement of the spleen, liver and kidney associated with yellowish ascites. Numerous white nodules as found in naturally infected case were also observed in the spleen, liver and kidney.

Bacterial re-isolation

Acid-fast bacteria were isolated from all samples of experiment group on 1% Ogawa media. Their major biological and biochemical characteristics corresponded to those of M. marinum according to the textbooks mentioned above, and to those of original strain (Weerakhun et al., 2007).

Histopathology

1. Experiment group

After one week, severe myonecrosis of large areas of the myotome developed on the injected side. The lesion was associated with eosinophilic edematous infiltration, moderate hemorrhaging and inflammatory cellular response, which mainly consisted of lymphocytes and macrophages following the line of the injection. Some of the necrotic areas were partly surrounded by epithelioid cells. While acid-fast bacteria were scattered over the lesions, they were apparently concentrated at the necrotic areas (Fig. 1). Large necrotizing areas were observed in the splenic parenchyma and the renal interstitium with concentration of acid-fast bacteria, however, encapsulation by epithelioid cells was not prominent around these lesions (Fig. 2). The bacteria were also sparsely observed in the hepatic parenchyma without inflammatory cellular response.

After two weeks, we observed chronic proliferative lesions composed of multiple caseous granulomas with surrounding granulation tissue at the injected site (Fig. 3). The granuloma was composed of a central caseous area and a surrounding layer of epithelioid cells, but no giant cells was observed in the lesions. Large granulomas
with irregular necrotic core were observed in the lesions, and it was considered that the large ones resulted from several caseous granulomas fused each other. The granulation tissue surrounding the granulomas was composed of lymphocytes, macrophages, fibroblasts, and newly generated myocytes and capillaries. Loose connective tissue existed in the boundary region between the granulomas and the granulation tissues, and the marginal region of the granulation tissues. In the central caseous area, many acid-fast bacteria were found. These bacteria were also observed in the epithelioid cells and in the macrophages of the granulation tissue. Although histological features found in the spleen, kidney and liver were similar to those in the fish after one week, acid-fast bacterial in these organs were more prominent in the samples after two weeks.

After three weeks, the caseous granulomas were extensively observed on the non-injected side (Fig. 4), and the granulation tissue widely surrounded the granulomas and extended into the non-injected side. Numerous epithelioid cell granulomas with central caseous areas were found in the spleen and kidney. The number of acid-fast bacteria in these lesions was considered to be increased in comparison with the samples after two weeks.

Fig. 1. One week after inoculation, severe myonecrosis associated with edematic infiltration, moderate hemorrhaging and inflammatory cellular response were found following the line of the injection. Acid-fast bacteria were apparently concentrated at the necrotic areas (arrow). ZN. Bar=200 µm.

Fig. 2. Large necrotizing areas were observed in the splenic parenchyma with concentration of acid-fast bacteria (arrows). ZN. Bar=200 µm.

Fig. 3. Two weeks after inoculation, chronic proliferative lesions composed of multiple caseous granulomas with surrounding granulation tissue were observed at the injected site (arrows). ZN. Bar=200 µm.

Fig. 4. Three weeks after inoculation, the caseous granulomas were extensively observed on the non-injected side (arrows). HE. Bar=200 µm.
No lesions associated with acid-fast bacteria were detected in the gill tissue examined in this study.

2. Control group

After one week, tissue damage caused by the passage of the needle inserting the inoculum and difference of osmotic pressure between the inoculum and tissue fluid was observed following the line of the injection. The damage consisted of small areas of hemorrhage with mild inflammatory infiltration and a small amount of degenerated muscle fibers. Myophagia of degenerated muscle fibers was observed in all specimens.

After two weeks, numerous regenerated muscle fibers obviously developed in the traumatic lesion. After three weeks, the traumatic lesion was entirely repaired by the regenerated muscle fibers.

No lesions were observed in the spleen, kidney, liver and gills collected from this group.

Discussion

In this study, histopathological features found in the fish of two and three weeks after inoculation were quite similar to those in the naturally infected fish (Weerakhun et al. 2007). In addition, the strain could cause disseminated granulomatous myositis, granulomatous splenitis and granulomatous interstitial nephritis that were described as the major histopathological features in the naturally infected case (Weerakhun et al. 2007). In light of these findings, the strain used in this study was considered to be a causative agent of mycobacteriosis found in cultured yellowtail at Kagoshima Prefecture in 2004.

Gross findings observed in the fish examined in this study corresponded with those in cultured yellowtail infected with Mycobacterium sp. (Kusuda et al. 1987). In addition, histopathological features in the fish examined resembled those in mycobacterioses of freshwater and marine fishes caused by Mycobacterium spp. (Mori et al. 1986; Chinabut et al. 1990; Hatai et al. 1993), M. marinum (Noga et al. 1989) and M. chelonae (Daoust et al. 1989; Brocklebank et al. 2003). In the light of these findings, it is likely that each mycobacterial species does not cause specific gross and histopathological features.

Some strains of M. marinum have been reported to cause cutaneous infection in human being (Arai et al. 1984; Ang et al. 2000) and recently studied with special emphasis on opportunistic infection among persons infected with the human immunodeficiency virus (Glaser et al. 1994; Angulo et al. 1994; Bartralot et al. 2000). With concerns about zoonotic problems, pathogenicity of the bacterial strain used in this study against mammals should be revealed because it was isolated from an important food fish species in Japan.

References


養殖ブリから分離された Mycobacterium marinum の人為感染試験

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養殖ブリから分離された Mycobacterium marinum の病原性を確認するため人為感染試験を実施した。人為感染魚にみられた肉眼所見は養殖ブリの Mycobacterium sp. 感染症と一致し、菌接種後 2 週間および 3 週間で観察された病理組織学的所見は自然発生例にみられたものと同一であった。以上のことから、供試した菌株は自然発生例の原因菌であると判断された。