Research article

Prevalence of Infection with *Nocardia seriolae* in Juvenile of Yellowtail *Seriola quinqueradiata* Cultured in Owase Bay, Japan

Goshi Kato^{1*}, Kensuke Oka¹, Megumi Matsumoto¹, Motohisa Kanemaru²,
Masato Yamamoto² and Motohiko Sano¹

¹Department of Marine Biosciences, Tokyo University of Marine Science and Technology,
Tokyo 108-8477, Japan

²OWASEBUSSAN Co., Ltd., Mie 519-3612, Japan

(Received August 30, 2019)

ABSTRACT—Nocardiosis caused by *Nocardia seriolae* makes severe losses of fish production of *Seriola* spp. in Japan. In this study, we investigated prevalence of *N. seriolae* infection in yellowtail *Seriola quinqueradiata* cultured in an open sea net-cage in Owase Bay, Japan. The gills, serum and spleen were sampled from juvenile fish ($n = 5$) on May, June, July, August, November 2017 and January 2018. Serum antibody titer against *N. seriolae* were significantly increased on November 2017 and January 2018. *N. seriolae* 16S rRNA gene were detected by PCR in the spleen and/or gills of 1–4 individuals sampled on May, July, August, November 2017 and January 2018, while the bacterium was not isolated from any individuals on Ogawa medium. Granulomatous inflammation which is the typical characteristic of pathological feature of nocardiosis was not observed in any spleen sections examined. However, fluorescent immunohistochemistry with anti-*N. seriolae* rabbit serum detected the long- and short-rod shaped bacteria in the spleen of all fish examined in this study. These data suggest that the juvenile yellowtail cultured in Owase Bay had been already infected with *N. seriolae* even in May. In addition, *N. seriolae* may be in viable but non-culturable state (VBNC), resulting in latent infection in cultured yellowtail.

Key words: *Nocardia seriolae*, Nocardiosis, *Seriola quinqueradiata*, yellowtail, prevalence, VBNC, latent infection

Nocardia seriolae is a Gram-positive, weakly acid-fast and nonmotile actinomycete which causes nocardiosis in cultured fish such as yellowtail *Seriola quinqueradiata* and amberjack *S. dumerili* (Kariya *et al.*, 1968; Kusuda and Taki, 1973; Kudo *et al.*, 1988). The disease first recognized in yellowtail and amberjack cultured in Owase Bay, Mie Prefecture, Japan (Kariya *et al.*, 1968) and then spread to Southern Japan where *Seriola* fish were actively cultured (Kusuda and Nakagawa, 1978; Matsuzato, 1978). To date, two antibiotics, sulfisoxazole sodium and sulfamonomethoxine has been approved for use in *Seriola* fish culture for the treatment of nocardiosis in Japan (Itano and Kawakami, 2002; Ismail *et al.*, 2012). However, outbreaks of the diseases are reported every year in aquaculture of *Seriola* fish species, and the disease causes severe economic losses in the fish production in Japan.

Nocardia seriolae slowly multiplies in the host and

causes a chronic progression of symptoms ending in death (Kariya *et al.*, 1968). The diseased fish externally shows multiple ulcers/nodules on the skin (Kariya *et al.*, 1968). Internally, hypertrophy of the spleen and kidney, abscesses in the epidermis and formation of tubercles in the spleen and kidney are observed in the diseased fish (Kariya *et al.*, 1968; Kubota *et al.*, 1968). In addition, there are cases in which the formation of tubercles was also observed in the gills of diseased fish (Kusuda *et al.*, 1974). The large abscesses observed in the spleen and subcutaneous adipose tissue was histopathologically characterized by marked infiltration of neutrophils (Miyazaki, 1980). The small nodules/tubercles observed in the spleen and kidney consists of epithelioid cells surrounding the central necrotic area (Kubota *et al.*, 1968; Miyazaki, 1980). These characteristic symptoms have been also observed in other cultured fish infected with *N. seriolae*, such as jade perch *Scortum barcoo* (Wang *et al.*, 2017) and pompano *Trachinotus blochii* (Labrie *et al.*, 2008).

The nocardiosis tends to be prevalent from summer

* Corresponding author
E-mail: gkato00@kaiyodai.ac.jp

to autumn with high water temperature in cultured yellowtail (Kariya *et al.*, 1968; Kusuda and Nakagawa, 1978). Consistent with the high prevalence in summer season, the optimum growth temperature of *N. seriolae* has been reported to be 25°C (Kariya *et al.*, 1968). Kusuda and Nakagawa (1978) investigated the bacterial number of *N. seriolae* in the sea water sampled in Nomi Bay, Kochi Prefecture, and reported that a large number of the bacteria were detected from August to October. Furthermore, serum antibody titer against *N. seriolae* significantly increased from August to January of the next year in yellowtail cultured in Ehime Prefecture (Itano *et al.*, 2008). These results suggest that infection of *N. seriolae* initiate around summer season in cultured *Seriola* fish species.

In this study, we investigated prevalence of *Nocardia seriolae* in apparently healthy yellowtail juvenile cultured in an open sea net-cage in Owase Bay, Japan. We first determined specific antibody titer against *N. seriolae* by ELISA and attempted to detect the bacteria by bacterial isolation. Species specific PCR reported by Miyoshi and Suzuki (2003) were also performed to detect *N. seriolae* from the spleen and gills. Further, immunohistochemistry using rabbit antiserum against *N. seriolae* was carried out to reveal the state of the bacteria in the spleen of apparent healthy individuals.

Materials and Methods

Sampling period

Yellowtail *S. quinqueradiata* juvenile fish ($n = 5$) introduced in an open sea net-cage on May 21 were sampled in Owase bay on May 29, June 26, July 24, August 29, November 20 in 2017 and January 25 in 2018. Body weight of the sampled fish was recorded at each time point except for fish sampled at November 20, 2017.

Enzyme-linked immunosorbent assay (ELISA)

Blood was removed by caudal venous puncture, allowed to clot at 4°C overnight and the serum was separated by centrifugation at 4,600 $\times g$ for 10 min. The serum was kept in -30°C until use. *Nocardia seriolae* strain TUMSAT-NS001 isolated from diseased yellowtail (Kato *et al.*, 2012) was cultured on 1% Ogawa medium (Nissui) at 25°C for 3 weeks. The colonies on the medium (10 mg wet weight) were scraped and suspended in 1 mL of sterile phosphate buffered saline [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH = 7.4, PBS (-)] using a glass homogenizer. After several times of freeze-thaw cycle, the bacterial cell suspension was sonicated for 4 min (a 30 s sonication and a 30 s cooling on ice). Each well of a 96-well plate (ELISA plate H, Sumitomo Bakelite) was filled with 25 mM carbonate buffer (pH 9.6) containing 5 mg/mL bacterial

antigens and incubated overnight at 4°C. After removing the antigen solution, each well of the 96-well plate was blocked with 1% skim milk in tris buffered saline containing 0.5% tween 20 (TBST) for 2 h at 4°C. After washing three times with TBST, the serum samples diluted 1:20 with the blocking solution was added to wells and incubated for 1 h at 4°C. The 96-well plate was washed three times with TBST and incubated with rabbit antiserum raised against yellowtail immunoglobulin (kindly provided by Dr. Ikuo Hirono) diluted 1:2,000 with the blocking solution for 1 h at 4°C. Following three times washing with TBST, the 96-well plate was incubated with goat anti-rabbit IgG secondary antibody HRP conjugated (Rockland Immunochemicals) diluted 1:4,000 with the blocking solution. The plate was washed as well and incubated with SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL) for 1.5 min at room temperature following manufacturer's instructions. Finally, 1 N HCl was added to each well to stop the reaction and absorbance (450 nm) was measured using MPR-A4i (TOSO).

Isolation of *N. seriolae*

The spleen was weighed, homogenized in sterile PBS (-) using mortar and pestle, and adjusted 50 mg/mL. One hundred micro litter of the suspension was inoculated on 1% Ogawa medium and incubated for 4 weeks at 25°C.

Detection of *N. seriolae* by PCR

DNA extraction and PCR to detect *N. seriolae* was performed following the protocol by Miyoshi and Suzuki (2003). A piece of the spleen or gills (20 mg) was homogenized in 100 μ L of sterile PBS (-) as above and incubated with 100 μ g of proteinase K for 1 h at 65°C. PBS (-) supplemented with 0.05% Tween 80 was added to the sample up to 1 mL, mixed well and the sample was centrifugated at 14,000 $\times g$ for 10 min. The pellet was resuspended in tris EDTA saline (TES, 50 mM Tris-HCl; 50 mM NaCl; 5 mM EDTA) supplemented with 1% sodium dodecyl sulfate and 200 μ g of proteinase K, and incubated for 1 h at 65°C. DNA was extracted using phenol: chloroform: isoamylalcohol = 25: 24: 1, precipitated by 99.9% ethanol, dried in air and solved in 13 μ L of distilled water. Reaction mixture contained 1 μ L of DNA sample, 0.6 μ L of the NS1 and NG1 primer (10 μ M) for 16s rRNA gene of *N. seriolae* (Miyoshi and Suzuki, 2003), 3 μ L of dNTP mixture (2.5 mM), 10 $\times$ Ex Taq buffer (TAKARA BIO) and Ex Taq DNA polymerase (TAKARA BIO) were adjusted up to 30 μ L with distilled water. PCR was carried out for 42 cycles of 94°C for 1 min, 57°C for 20 s and 72°C for 1 min with a final extension of 72°C for 10 min. PCR products were electrophoresed on a 1% agarose gel at 100 V for 10 min and the gel was stained with Midori Green Advance (Nippon Genetics). The gel images were captured under UV

light with a digital camera COOLPIX P7800 (Nikon) and analyzed using Adobe Photoshop CS6 (Adobe Systems). In addition, PCR products were cloned into pGEM T-easy vector (Promega) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) with a 3130xl genetic analyzer (Applied Biosystems).

Histology and Immunohistochemistry

A piece of the spleen (approximately 5 mm square) was fixed in Davidson's fixative for 24 h at room temperature, embedded in paraffin and sectioned at 3 μ m. The sections were deparaffinized and stained with hematoxylin and eosin (HE). For immunohistochemistry, deparaffinized sections were blocked with 1% skim milk in TBS for 1 h at 4°C and washed three times with TBS. Anti-*N. seriolae* rabbit serum (kindly provided by Dr. Terutoyo Yoshida) diluted 1: 400 with the blocking solution was added onto the sections and the sections were incubated for 1 h at 4°C. In addition, deparaffinized sections incubated with 1: 400 dilution of normal rabbit serum was used as a negative control. After three times washing with TBS, the slides were stained with 3,3-diaminobenzidine (DAB) using VECTASTAIN ABC Rabbit IgG Kit (Vector Laboratories), following manufacturer's instructions. The DAB stained sections were then counterstained with hematoxylin. Furthermore, the sections incubated with the primary antibody were also stained with a 1: 500 dilution of goat anti-rabbit IgG AlexaFluor488 (Thermo Fisher Scientific) in the blocking solution, for 1 h at 4°C. The sections were washed three times with TBS and counterstained with Hoechst 33342 (Thermo Fisher Scientific). Digital images were captured using a Nikon ECLIPSE Ti S fluorescence microscope (Nikon) and analyzed using NIS Elements software (Nikon). The number of bacteria on a section (mean area $\pm$ S.D. = 8.6 ± 3.1 mm²) was counted under the fluorescent microscopy and infection intensity was assessed as weekly positive (+, >10); positive (++, >50) and strongly positive (+++, >100).

Mycobacterium sp. TUMSAT Msp-001 isolated from cultured yellowtail (Kato *et al.*, 2010) were grown on the Ogawa medium. The colonies were scraped and smeared on a slide glass and the slide glass was subjected to the fluorescent immunohistochemistry to check cross reactivity of the rabbit serum.

Statistics

Results were calculated as the mean value of five individuals. Significant differences among the sampling groups were assessed using one-way analysis of variance (ANOVA) and Tukey's *post-hoc* test. $P < 0.05$ were considered statistically significant.

Results

Changes of body weight of sampled fish and bacterial isolation

Yellowtail cultured in the cage grew up as usual, body weight of the sampled fish increased over the experimental period (Fig. 1). No mortality of cultured yellowtail including juveniles and yearling fish due to nocardiosis was observed in Owase Bay during the sampling period. No colony was found from any samples on 1% Ogawa medium after the incubation (data not shown).

ELISA

The serum antibody titers against *N. seriolae* were significantly higher in November 20 and January 25 than the other time points (Fig. 2).

Fig. 1. Body weight of yellowtail sampled in the Owase bay. Different characters show significant differences. N.A. = not applicable.

Fig. 2. Antibody titer in the serum of yellowtail sampled in the Owase bay. Different characters show significant differences.

Fig. 3. PCR detection of *Nocardia seriolae* 16s rRNA gene from the spleen (A) and the gills (B) of yellowtail sampled in the Owase bay. Arrowheads show the amplicon corresponding to 432 bp target sequence. Different characters show significant differences.

PCR

The number of individuals positive for *N. seriolae* by the PCR increased from July 24 in both of the spleen and gills. A PCR amplicon of the expected size (432 bp) were detected in the kidney from one fish on May 29, three fish on June 26, two fish on July 24, two fish on August 29, one fish on November 20 and three fish on January 26 (Fig. 3A). While the corresponding PCR bands were detected in the gills from three fish on July 24, three fish on August 29, four fish on November 20 and three fish on January 26 (Fig. 3B).

Histology and immunohistochemistry

In this study, granulomatous inflammation which is the typical characteristic of pathological feature of nocardiosis was not observed in any spleen sections examined (Fig. 4A). However, fluorescence immunohistochemistry detected the long- and short-rod shaped bacteria in the spleen of almost all fish examined (Fig. 4B-D). Furthermore, several bacterial aggregations were also detected on the sections in immunohistochemistry with DAB staining (Fig. 4C). Long- and short-rod shaped bacteria corresponding to the fluorescent immunohistochemistry were found in the bacterial

aggregations in the high magnification (Fig. 4D). Infection intensities assessed by the number of bacteria per a section were shown in Table 1. The spleen section strongly positive for *N. seriolae* was frequently observed on January 25, while the intensity values were relatively low on May 29 and June 26.

No fluorescent signal was observed on the smeared *Mycobacterium* sp. on the glass slide and the sections incubated with normal rabbit serum.

Discussion

Previous studies suggest that nocardiosis of cultured yellowtail began between July and August, based on the antibody titers (Itano *et al.*, 2008). In this study, antibody titer of juvenile fish was higher on November and January than the other time points. Further, *N. seriolae* were detected from all samples examined in the fluorescent immunohistochemistry and several individuals were also positive for *N. seriolae* in the PCR detection. However, neither external nor internal symptoms were observed in all juvenile fish examined in this study. *Nocardia farcinica* and other pathogenic *Nocardia* spp. often cause asymptomatic latent infection

Fig. 4. Histopathology and immunohistochemistry with anti-*N. seriola* rabbit serum in the spleen of yellowtail sampled in the Owase bay. (A) Histopathology in the spleen of yellowtail sampled on May 29 (Fish No. 2). H&E staining. Bar = 100 μ m. (B-D) Fluorescent immunohistochemistry with anti-*N. seriola* rabbit serum in the spleen of yellowtail sampled on May 29 (Fish No. 2, B), on June 26 (Fish No. 4, C) and on May 29 (Fish No. 3, D). Bar = 10 μ m. (E) Immunohistochemistry with DAB staining in the spleen of yellowtail sampled on July 24 (E, Fish No. 2). Arrowheads show bacterial aggregations. m, melano-macrophage center. Bar = 100 μ m. (F) High magnification in the corresponding area to the squared area in E. Bar = 10 μ m.

Table 1. PCR detection of *Nocardia seriolae* and the number of bacteria per a section of the spleen in yearling yellowtail

Fish	May 29			June 26			July 24			August 29			November 20			January 25		
	PCR ^a	PCR ^b	FIHC ^c	PCR ^a	PCR ^b	FIHC ^c	PCR ^a	PCR ^b	FIHC ^c	PCR ^a	PCR ^b	FIHC ^c	PCR ^a	PCR ^b	FIHC ^c	PCR ^a	PCR ^b	FIHC ^c
1	–	–	–	–	–	+	+	+	+	–	–	+	–	+	++	–	–	+++
2	+	–	++	–	–	+	+	–	+++	–	–	+	+	–	++	–	–	++
3	–	–	+	–	–	+	+	+	–	–	+	++	–	+	++	+	+	+++
4	–	–	++	–	–	+	+	–	+	+	+	+	–	+	+	+	+	+
5	–	–	+	–	–	+	–	+	+	+	+	+	+	+	–	+	+	++

^a PCR using the spleen samples

^b PCR using the gill samples

^c FIHC, fluorescent immunohistochemistry; + >10, ++ >50, +++ >100

in immunocompetent patients in human (Shimada and Iwai, 2010). While, severely immunocompromised patients show the progressive, disseminated and life-threatening infection by *Nocardia* spp. (McNeil and Brown, 1994). *M. tuberculosis* also cause a latent infection, and the patients with latent tuberculosis infection show high antibody titer against ESAT6 (antigen expressed in the growth phase) and MDP1 (antigen expressed in the dormant phase) (Maekura *et al.*, 2019). Present results showed that the juvenile fish cultured in the Owase Bay had been already infected with *N. seriolae* on May 29 and *N. seriolae* may also cause latent infection in seriola fish. Yellowtail latently infected with *N. seriolae* might turn out to be the progressive infection when their immune activities are suppressed.

Although long- and short-rod shaped bacteria were detected in the spleen of almost all fish examined in immunohistochemistry, no bacterial colony was observed on the Ogawa medium inoculated with the spleen sample. Many bacterial species have been found to be a viable but non-culturable (VBNC) state under environmental stresses (Li *et al.*, 2014). Pathogenic mycobacteria are known to enter VBNC state during infection of the host in mammals. *M. tuberculosis* and *M. smegmatis* enters VBNC state under starvation, oxygen limitation and altered temperature during the infection (Kuznetsov *et al.*, 2004; Shleeva *et al.*, 2004; Downing *et al.*, 2005). The VBNC state, characterized by low metabolic activity with non- or low-replicating persistence, contributes to their survival in latent tuberculosis in human host (Gengenbacher and Kaufmann, 2012). When *M. tuberculosis* is VBNC state in the host, it does not cause any diagnosable symptoms until reactivation (Li *et al.*, 2014). After entering the VBNC state, *Rhodococcus biphenylivorans* cells changed from rods to short-rods or cocci and they were significantly smaller than exponential-phase cells (Su *et al.*, 2015). Nitrate reduction pathway are activated in dormant stage of *M. tuberculosis* and the pathway plays respiratory function in the absence of oxygen (Khan and Sarkar, 2012; Akhtar *et al.*, 2013). Nitrate reductase genes *narG*, *narH*, *narI*, *narJ*, *nirB* and *nirD* that included in the nitrate reduction pathway are also found in *N. seriolae* genome (Yasuike *et al.*, 2017). Taken together, *N. seriolae* detected in apparently healthy yellowtail in this study may be VBNC state, resulting in latent infection in the fish.

The number of individuals that are positive for *N. seriolae* in PCR and strongly positive in fluorescent immunohistochemistry tended to increase November 2017 and January 2018. However, bacterial colony was not isolated even from none of the spleen sample on November 2017 and January 2018. Generally, *N. seriolae* are isolated using 1% or 3% Ogawa medium from diseased fish showing the symptoms of nocardiosis (Kariya *et al.*, 1968; Kusuda and Taki, 1973; Kusuda *et al.*, 1974; Itano *et al.*, 2002). Bacteria entered to VBNC

state can be cultivated only under the resuscitation condition, but not under the normal condition (Li *et al.*, 2014). These results suggest that the bacteria detected in the spleen of apparently healthy juvenile fish were still VBNC state in the host during the winter season. The increase of *N. seriolae* in juvenile fish during winter season may be caused by uptake and accumulation of the bacteria from environmental seawater, rather than bacterial proliferation in the fish.

With a few exceptions, the individual strongly positive for *N. seriolae* in the fluorescent immunohistochemistry were also positive in the PCR detection. While, *N. seriolae* was almost not detected by the PCR in the individuals weakly positive for *N. seriolae* in the fluorescent immunohistochemistry. *Nocardia* species have a thick cell wall structure contains complex waxes and glycolipids, so that DNA extraction from the bacterium is extremely difficult. Several papers introduce new techniques for DNA extraction from *Nocardia* spp. such as sodium chloride-TRIS-EDTA-triton method (Bafghi *et al.*, 2014) and microwave oven method (Faghri *et al.*, 2014). These techniques may contribute better PCR sensitivity to detect *N. seriolae* in apparent healthy yellowtail.

In conclusions, the juvenile yellowtail cultured in the Owase Bay had been already infected with *N. seriolae* even immediately after the introduction into the marine cage. In these infected but apparently healthy fish, *N. seriolae* may be in VBNC state and cause latent infection. The present study revealed the actual prevalence of *N. seriolae* infection in yellowtail cultured in Owase Bay, as an example. Further epidemiological survey is needed to understand the perspective of the prevalence of *N. seriolae* infection in cultured yellowtail.

Acknowledgments

We would like to thank Dr. Ikuo Hirono for giving us the rabbit antiserum raised against yellowtail immunoglobulin. We would also like to thank Dr. Terutoyo Yoshida for giving us the Anti-*N. seriolae* rabbit serum.

References

- Akhtar, S., A. Khan, C. D. Sohaskey, C. Jagannath and D. Sarkar (2013): Nitrite reductase NirBD is induced and plays an important role during *in vitro* dormancy of *Mycobacterium tuberculosis*. *J. Bacteriol.*, **195**, 4592–4599.
- Bafghi, M. F., S. S. Eshraghi, P. Heidarieh, S. Habibnia and M. R. Nasab (2014): DNA extraction from nocardia species for special genes analysis using PCR. *N. Am. J. Med. Sci.*, **6**, 231–233.
- Downing, K. J., V. V. Mischenko, M. O. Shleeva, D. I. Young, M. Young, A. S. Kaprelyants, A. S. Apt and V. Mizrahi (2005): Mutants of *Mycobacterium tuberculosis* lacking three of the five *rpf*-Like genes are defective for growth *in vivo* and for resuscitation *in vitro*. *Infect. Immun.*, **73**, 3038–3043.
- Faghri, J., S. Bourbour, S. Moghim, M. Meidani, H. G. Safaei, N. Hosseini, B. N. Esfahani, H. Fazeli and M. Sedighi

- (2014): Comparison of three phenotypic and deoxyribonucleic acid extraction methods for isolation and Identification of *Nocardia* spp. *Adv. Biomed. Res.*, **3**, 151.
- Ismail, T. F., A. Nakamura, K. Nakanishi, T. Minami, T. Murase, S. Yanagi, T. Itami and T. Yoshida (2012): Modified resazurin microtiter assay for in vitro and in vivo assessment of sulfamonomethoxine activity against the fish pathogen *Nocardia seriolae*. *Fish Sci.*, **78**, 351–357.
- Itano, K. and H. Kawakami (2002): Drug susceptibility of recent isolates of *Nocardia seriolae* from cultured fish. *Fish Pathol.*, **37**, 152–153. (In Japanese with English summary)
- Kariya, T., S. Kubota, Y. Nakamura and K. Kira (1968): Nocardial infection in cultured yellowtails (*Seriola quinqueriata* and *S. purpurascens*)—I. *Fish Pathol.*, **3**, 16–23. (In Japanese)
- Kato, G., H. Kondo, T. Aoki and I. Hirono (2010): BCG vaccine confers adaptive immunity against *Mycobacterium* sp. infection in fish. *Dev. Comp. Immunol.*, **34**, 133–140.
- Kato, G., H. Kondo, T. Aoki and I. Hirono (2012): *Mycobacterium bovis* BCG vaccine induces non-specific immune responses in Japanese flounder against *Nocardia seriolae*. *Fish Shellfish Immunol.*, **33**, 243–250.
- Kubota, S., T. Kariya, Y. Nakamura and K. Kira (1968): Nocardial infection in cultured yellowtails (*Seriola quinqueriata* and *S. purpurascens*)—II. *Fish Pathol.*, **3**, 24–33. (In Japanese)
- Kudo, T., K. Hatai and A. Seino (1988): *Nocardia seriolae* sp. nov. causing nocardiosis of cultured fish. *Int. J. Syst. Bacteriol.*, **38**, 173–178.
- Khan, A. and S. Dhiman (2012): Nitrate reduction pathways in mycobacteria and their implications during latency. *Microbiology*, **158**, 301–307.
- Kusuda, R. and A. Nakagawa (1978): Nocardial infection of cultured yellowtail. *Fish Pathol.*, **13**, 25–31. (In Japanese with English summary)
- Kusuda, R. and H. Taki (1973): Studies on a Nocardial infection of cultured yellowtail—I. Morphological and biochemical characteristics of *Nocardia* isolated from diseased fishes. *Nippon Suisan Gakkaishi*, **39**, 937–943. (In Japanese with English summary)
- Kusuda, R., H. Taki and T. Takeuchi (1974): Studies on a Nocardial infection of cultured yellowtail—II. Characteristics of *Nocardia kampachi* isolated from a gill-tuberculosis of yellowtail. *Nippon Suisan Gakkaishi*, **40**, 369–373. (In Japanese with English summary)
- Kuznetsov, B. A., M. E. Davydova, M. O. Shleeva, S. V. Shleev, A. S. Kaprelyants and A. I. Yaropolov (2004): Electrochemical investigation of the dynamics of *Mycobacterium smegmatis* cells' transformation to dormant, nonculturable form. *Bioelectrochemistry*, **64**, 125–131.
- Gengenbacher, M. and S. H. E. Kaufmann (2012): *Mycobacterium tuberculosis*: success through dormancy. *FEMS Microbiol. Rev.*, **36**, 514–532.
- Labrie, L., J. Ng, Z. Tan, C. Komar, E. Ho and L. Grisez (2008): Nocardial infections in fish: An emerging problem in both freshwater and marine aquaculture systems in Asia. In "Diseases in Asian Aquaculture VI" (ed. by M. G. Bondad-Reantaso, C. V. Mohan, M. Crumlish and R. P. Subasinghe). Fish Health Section, Asian Fisheries Society, pp. 297–312.
- Li, L., N. Mendis, H. Trigui, J. D. Oliver and S. P. Faucher (2014): The importance of the viable but non-culturable state in human bacterial pathogens. *Front. Microbiol.*, **5**, 258.
- Maekura, R., S. Kitada, M. Osada-Oka, Y. Tateishi, Y. Ozeki, T. Fujikawa, M. Miki, J. Ogawa, M. Mori and S. Matsumoto (2019): Serum antibody profiles in individuals with latent *Mycobacterium tuberculosis* infection. *Microbiol. Immunol.*, **63**, 130–138.
- Matsuzato, T. (1978): Nocardiosis of cultured yellowtail. *Fish Pathol.*, **13**, 33–34. (In Japanese with English summary)
- McNeil, M. M. and J. M. Brown (1994): The medically important aerobic actinomycetes: epidemiology and microbiology. *Clin. Microbiol. Rev.*, **7**, 357–417.
- Miyazaki, T. (1980): Histopathological study on bacterial infections in fishes. *Bulletin of the Faculty of Fisheries, Mie University*, **7**, 63–149. (In Japanese with English summary)
- Shimada, S. and K. Iwai (2010): Subcutaneous abscess due to *Nocardia farcinica*. *J. J. Inf. D.*, **84**, 206–209. (In Japanese with English summary)
- Shleeva, M., A. Goncharenko, Y. Kudykina, D. Young, M. Young and A. Kaprelyants (2013): Cyclic Amp-dependent resuscitation of dormant mycobacteria by exogenous free fatty acids. *PLoS ONE*, **8**, e82914.
- Su, X., F. Sun, Y. Wang, M. Z. Hashmi, L. Guo, L. Ding and C. Shen (2015): Identification, characterization and molecular analysis of the viable but nonculturable *Rhodococcus biphenylivorans*. *Scientific Rep.*, **5**, 18590.
- Wang, F., X. Wang, C. Liu, O. Chang, Y. Feng, L. Jiang and K. Li (2017): *Nocardia seriolae* infection in cultured jade perch, *Scortum barcoo*. *Aquacult. Int.*, **25**, 2201–2212.
- Yasuike, M., I. Nishiki, Y. Iwasaki, Y. Nakamura, A. Fujiwara, Y. Shimahara, T. Kamaishi, T. Yoshida, S. Nagai, T. Kobayashi and M. Katoh (2017): Analysis of the complete genome sequence of *Nocardia seriolae* UTF1, the causative agent of fish nocardiosis: The first reference genome sequence of the fish pathogenic *Nocardia* species. *PLoS One*, **12**, e0173198.