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DISEASES CAUSED BY BACTERIAL PATHOGENS IN SALTWATER

Yukinori Takahashi
Department of Applied Aquabiology, National Fisheries University, 2-7-1 Nagata-Honmachi Shimonoseki, Yamaguchi 759-6595, Japan.

Terutoyo Yoshida, Issei Nishiki
Department of Aquaculture, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan.

Masahiro Sakai
Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan.

Kim D. Thompson, Alexandra Adams
Institute of Aquaculture, University of Stirling, Stirling, Stirlingshire, FK9 4LA, Scotland, UK.

Tae-Sung Jung
Aquatic Biotechnology Center of WCU Project, College of Veterinary Medicine, Gyeongsang National University, Jinju, Gyeongnam 660-710, South Korea.

Takashi Aoki
Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, 513, Wasedatsurumaki-cho, Shinjuku-ku, Tokyo 162-0041, Japan.

Hisatsugu Wakabayashi
Emeritus Professor, the University of Tokyo, 1688 Futatsugi, Matsudo, Chiba 270-0027, Japan.

Jun-ichi Hikima
Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, Miyazaki, Japan

Tomokazu Takano
Aquatic Animal Health Division, National Research Institute of Aquaculture, Fisheries Research Agency, 422-1 Nakatsuhamaura, Minami-ise, Mie 516-0193, Japan.

Takaji Iida
Japan Sea National Fisheries Research Institute, Fisheries Research Agency, 422-1 Nakatsuhamaura, Minami-ise, Mie 516-0193, Japan.

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Vibriosis, Gram-posistive bacteria, virulence factors, vaccine, Tenacibaculosis, Tenacibaculum maritimum, Tenacibaculum ovoliticum. gliding bacteria, Edwardsiella tarda, septicaemia, yellowtail, bacterial infection, jaundice.

Contents

Summary

This chapter also summarized eight different fish diseases from saline water, viz. 1) Saltwater Streptococcosis (Streptococcus Dysgalactiae, Streptococcus Iniae, Streptococcus parauberis), 2) Nocardiosis, 3) Mycobacterial disease, 4) Pasteurellosis, 5) Vibriosis, 6) Tenacibaculosis, 7) Edwardsiellosis (*Edwardsiella tarda*), and 8) bacterial hemolytic jaundice. The characteristics of disease agent, and pathogenesis, histopathological interest, diagnostic method, chemotherapy and disease control were introduced.

1. SALTWATER STREPTOCOCCOSIS

Terutoyo Yoshida

1.1. Lactococcus Garvieae

1.1.1. Abstract

Lactococcus garvieae infections occur in fish species cultured in saltwater and freshwater. Formerly, the causal agent isolated from diseased yellowtail, *Seriola quinqueradiata, L. garvieae* was classified as *Streptococcus* sp. in Japan. Later, the isolate was identified as a new species, *Enterococcus seriolicida*. Then, *E. seriolicida* was reclassified as a junior synonym of *L. garvieae*. One of virulence factors in *L. garvieae* is suspected to be a capsule with the resistance of opsono-phagocytosis in fish phagocytic cells. A variety of fish species including freshwater and saltwater fishes are susceptible to *L. garvieae* and effective vaccines have been developed to prevent *L. garvieae* infection.

1.1.2. Introduction

Lactococcus garvieae infections in fish species cultured in saltwater and freshwater have occurred. Formerly, the causal agent isolated from diseased yellowtail, *L. garvieae* was

classified as *Streptococcus* sp. in Japan (Kusuda *et al*., 1976). Later, the isolate was identified as a new species, *Enterococcus seriolicida* (Kusuda *et al*., 1991). Then, *E. seriolicida* was reclassified as a junior synonym of *L. garvieae* isolated from bovine mastitis (Teixeira *et al*., 1996).

L. garvieae was isolated from fish cultured in seawater and freshwater. *L. garvieae* was isolated from cultured fish and from domestic animals with mastitis, vegetables, and dairy foods (Kawanishi *et al*., 2007). Recently, *L. garvieae* has been isolated from humans with bacterial endocarditis (Watanabe *et al*., 2011). Although *L. garvieae* infection in Europe was observed mainly in freshwater fish, fish cultured in seawater such as yellowtail, amberjack, and king fish have been affected by *L. garvieae* infection in Japan. In this section, *L. garvieae* infection in cultured fish is defined as a causal agent in marine environments.

1.1.3. Disease Agent

1.1.3.1 Characteristics

L. garvieae is a Gram-positive, non-motile, non-spore-forming ovoid coccus that forms short chains in broth cultures and white colonies with α-hemolysis on blood agar. Isolates from fish grow in 6.5% NaCl broth at 45°C. Acid production was observed from sorbitol, mannitol, cellobiose, galactose, glucose, maltose, and trehalose, but not from adonitol, glycogen, and melibiose (Vendrell *et al*., 2006). The fish isolates could not assimilate lactose, as no gene was present for lactose utilization. In contrast, dairy isolates of *L. garvieae* could produce acid from lactose (Fortina *et al*., 2009). Its virulent strains were cell capsulated (Yoshida *et al*., 1997) with fimbriae (Ooyama *et al*., 2002). Figure 1.1.1 shows capsulated *L. garvieae*. Figure 1.1.2 shows a phase-contrast microscope image of well-developed cell capsulation of *L. garvieae*. *L. garvieae* can be isolated from diseased fish on tryptone soya agar, brain heart infusion agar, and Todd-Hewitt agar at 22°C−25°C for 48 h. Additional supplementation with NaCl is not required. Susceptibility of each strain from the genus *Seriola,* trout and terrestrial animals to three bacteriophages was investigated. All isolates from the genus *Seriola* were found to be susceptible to at least one of the bacteriophages. However, none of the isolates obtained from trout and terrestrial animals were susceptible to any of the three bacteriophages (Kawanishi *et al*., 2006). The epidemiological study revealed that *L. garvieae* isolates (n=427) from farmed fishes in Japan were very similar based on the analysis of biased sinusoidal gel electrophoresis separation of *Sma*-I digested fragments of genomic DNA (Nishiki *et al*., 2011). However, phenotypic heterogeneity and genetic diversity characterize *L. garvieae* strains isolated from diseased fish, cows, water buffalos, and humans (Vela *et al*., 2000).

Generally, the brain, spleen, and kidney of infected fish are recommended for bacterial isolation. Slide agglutination using an antiserum raised against capsulated *L. garvieae* is recommended for diagnosis (Yoshida *et al*., 1997).

1.1.3. 2 Genome Size

Genome sequences of *L. garvieae* (strain numbers LG9 and TB25) isolated from diseased rainbow trout and Italian Cheese revealed genomes of 20,877,027 and 2,012,328 bases,

respectively (Ricci *et al*., 2012). The results of the whole-genome shotgun sequencing projects were deposited at DDBJ/EMBL/GenBank with accession numbers AGQY00000000 (LG9) and AGQX00000000 (TB25). The complete genome sequence of *L. garvieae* isolated from yellowtail, *S. quinqueradiata,* contained 1,959,135 and 1,963,964 bases for ATCC49165 and Lg2, respectively (Morita *et al*., 2011). In the near future, comparative genome analyses of strains isolated from fish, humans, and other animals will reveal the relationship between virulence and phenotype in *L. garvieae.*

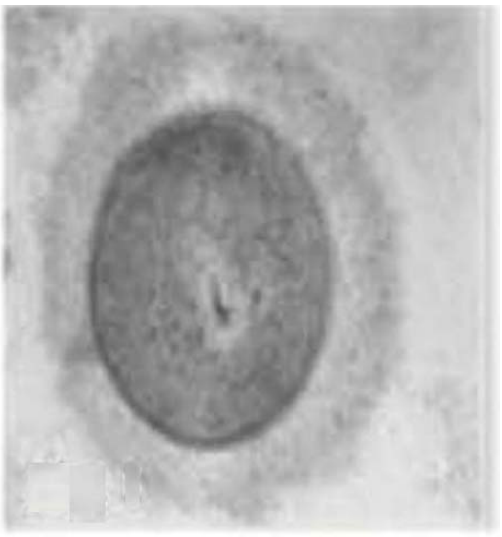

Figure 1.1.1. Capsulated virulent strain of *L. garvieae*

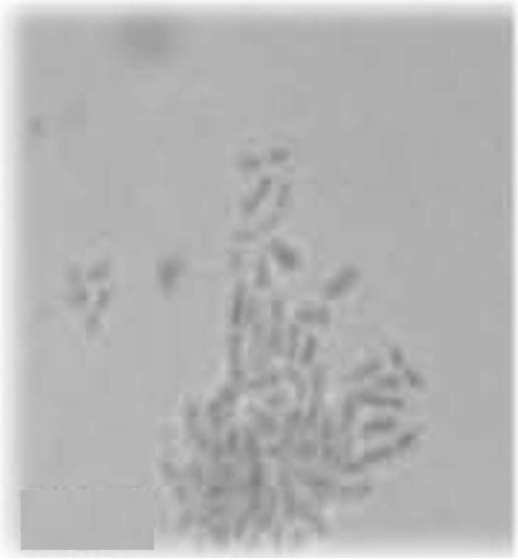

Figure 1.1.2. Phase contrast microscopy of well developed capsular strain of *L. garvieae*

1.1.3.3 Pathogenesis

Capsulated *L. garvieae* cells resist phagocytosis in fish but non-capsulated cells do not (Yoshida *et al*., 1997). *L. garvieae* isolated from the genus *Seriola* (*S. quinqueradiata*, *S. dumerili,* and *S. lalandi*) showed strong pathogenicity to yellowtail; *L. garvieae* isolated

from trout showed weak pathogenicity; and *L. garvieae* isolated from cows, pigs, cats, dogs, and horses showed no pathogenicity in fish (Kawanishi *et al*., 2006). *L. garvieae* strains isolated from diseased fish and animals showed no distinct pathogenicity to ddY mice (4-week-old female) with 10^8 cells injected intraperitoneally. The 50% lethal dose of virulent encapsulated cells was less than 1×10^2 cells per fish (Kawanishi *et al*., 2006). These results suggested that *L. garvieae* isolates from diseased fish has strong virulence to homogeneous fish species.

1.1.4. Diagnostic Methods

1.1.4.1 Serological Classification

Serological characterization of *L. garvieae* was performed using a slide agglutination technique. Antigenic types were designated as KG+ and KG-. The KG+ type could be agglutinated with antiserum raised against non-capsulated KG7409 strain, whereas the capsulated KG- type could not be agglutinated with the same antiserum. Several subcultures converted KG- strains into KG+ strains (Kitao *et al*., 1982). The both KG- and KG+ type strains could be agglutinated with antiserum raised against a capsulated type cell of KG- type. Transmission electron microscopy revealed a capsule on the cell surface of a KG- (non-agglutinating) type, but not on the cell surface of a KG+ (agglutinating) type (Yoshida *et al*., 1997).

1.1.4.2 Clinical Signs and Pathology

Fish infected with *L. garvieae* exhibited various clinical signs. Erratic swimming, whirling swimming, becoming dark in appearance, unilateral or bilateral exophthalmia (Figure 1.1.3), corneal opacity (Figure 1.1.4), and severe hemorrhage on the opercula were typical symptoms in infected fish. Sometimes, severe pericarditis and necrosis of the caudal peduncle were seen in infected fish. Clinical analysis is required to confirm the difference of diseases between *L. garvieae* and *Streptococcus dysgalactiae* infections in farmed yellowtail and amberjack due to the strong clinical similarities (Nomoto *et al*., 2004).

Figure 1.1.3. Typical symptom of infected yellowtail with exophthalmia

Figure 1.1.4. Infected amberjack showing corneal opacity

Figure 1.1.5. Vaccination (Injected vaccine) of *L. garvieae* for amberjack

1.1.4.3 PCR

A species-specific PCR technique for identification of *L. garvieae* from other similar bacteria was developed by targeting 16S rDNA (Zlotkin *et al*., 1998) or the dihydropteroate synthase gene (Aoki *et al*., 2000). Fortina *et al*. (2009) revealed that since the phospho-β-galactosidase gene could be detected in all strains isolated from dairy food but not in strains isolated from rainbow trout and catfish, this gene could be used as a reliable genetic marker to distinguish strains by their ability to assimilate lactose. A PCR assay based on the 16S-23S rRNA internal transcribed spacer region was developed to detect *L. garvieae* (Dang *et al*., 2012). A sensitive and specific LAMP (loop-mediated isothermal amplification) by using primers set designed from *L. garvieae* alpha/beta fold family gene was developed. The LAMP assay was 10 fold more sensitive than the PCR assay targeting 16S rDNA (Tsai *et al*., 2013).

1.1.5. Control

1.1.5.1 Pharmacotherapy

Antibiotic therapy can be used to treat *L. garvieae* (=*Streptococcus* sp.) infection (Aoki *et al*., 1990). Several effective antibiotics including macrolides (MLS), lincomycin (LCM), and tetracycline (TC) have been used to treat *L. garvieae* infection in Japan (Aoki *et al*., 1990). However, high-level resistance to these antibiotics was observed; strains resistant to MLS, LCM, and TC carried a transferable R-plasmid. Strains resistant to erythromycin (EM) and TC carried *erm*B and *tet*S, respectively (Maki *et al*., 2008).

1.1.5.2 Vaccine and Phage Therapy

A protective vaccine of injected or orally administered formalin-inactivated cells was developed for the genus *Seriola* (Ooyama *et al*., 1999). Figure 1.1.4 shows vaccination of amberjack at a farm site. Formalin-killed *L. garvieae* bacterin conferred long-term protection in yellowtail against artificial infection by encapsulated *L. garvieae* with long-lasting agglutinating titers against non-capsulated cells (Ooyama *et al*., 1999). A live attenuated capsule-deficient *L. garvieae* strain induced strong immune protection against virulent capsulated *L. garvieae* Ooyama *et al*., 2006). Therefore, possible immune-protective antigens had no capsule, but cell-surface antigens included cell-surface fimbriae (Ooyama *et al*., 2002). However, vaccinated rainbow trout only gained 3–6 months protection (Eldar *et al*., 1997).

The efficacy of phage therapy was verified by using lytic bacteriophages to treat experimentally infected yellowtail with *L. garvieae* (Nakai *et al*., 1999).

1.1.6. Recent Topics

Several researchers have reported the isolation of *L. garvieae* from the blood of human patients with endocarditis (Vinh *et al*., 2006; Watanabe *et al*., 2011). However, phenotypic heterogeneity and genetic diversity characterize *L. garvieae* strains isolated from diseased fish, cows, water buffalos, and humans (Vela *et al*., 2000).

1.2. Fish Pathogenic Lancefield Group C *Streptococcus Dysgalactiae*

Issei Nishiki and Terutoyo Yoshida

1.2.1. Synopsis

Streptococcus dysgalactiae is known mainly as a mammalian pathogen. This bacterium has been isolated from diseased fish in some Asian countries. The typical symptoms of *S. dysgalactiae* infection in farmed amberjack *Seriola dumerili* and yellowtail *S. quinqueradiata* include a severe necrosis in the caudal peduncle and pericarditis. Japanese fish isolates are genetically close to each other and distinguishable from mammalian isolates. Recently, *S. dysgalactiae* was isolated from blood culture of a patient who had handled raw fish, and the characteristics of this strain were the same as those of isolates from farmed fish. Therefore, *S. dysgalactiae* is a potential zoonotic pathogen causing economic loss and threatening public health. In Japan, a

formalin-inactivated vaccine against *S. dysgalactiae* has been commercially available since 2009.

Several years have passed since the first outbreak of *S. dysgalactiae* infection in Japanese fish farms in 2002. Recently, similar bacterial infections in farmed fish have been reported in other Asian countries as well as Japan. Similar bacterium was isolated from humans with cellulitis following preparation of fresh raw seafood. Zoonosis of these pathogens was suspected. This review described that the present status and information on *S. dysgalactiae* infection in fish.

1.2.2. Introduction

Since 2002, fish mortalities characterized by necrosis of the caudal peduncle have occurred in fish farms and α-hemolytic *Streptococcus* sp. was isolated from the necrotic lesions of diseased fish (Nomoto *et al*., 2004). Initially, these mortalities were thought to be caused by *L. garvieae* infection because of the strong clinical similarity to *L. garvieae* infection. Later, the isolated pathogen was identified as Lancefield group C *S. dysgalactiae* (Nomoto *et al*., 2004).

S. dysgalactiae (SD) belongs to Lancefield group C, G, and L serotypes, and is grouped with the pyogenic *Streptococcus*. SD is a well-known pathogen of animals and humans. Several studies have reported that SD causes mastitis in cattle (Aarestrup and Jensen, 1996), endocarditis in domestic animals, and cardiopulmonary diseases or adenoiditis in humans (Efstratious *et al*., 1994). Several fish species are susceptible to SD. In Japan, farmed amberjack *Seriola dumerili*, yellowtail *S. quinqueradiata,* and kingfish *S. lalandi* are all susceptible to SD (Nomoto *et al*., 2004 and 2006; Abdelsalam *et al*., 2010). In other Asian countries, SD has been isolated from gray mullet *Mugil cephalus*, basket mullet *Liza alata*, cobia *Rachycentron canadum,* and tilapia *Oreochromis* sp. (Abdelsalam *et al*., 2010). Although SD has been isolated from Amur sturgeon *Acipenser schrenckii* in China, no Lancefield sero-grouping of this pathogen has been reported (Yang and Li 2009). In Brazil, Lancefield group C SD has been isolated from Nile tilapia *Oreochromis niloticus*. A similar bacterium was isolated from a patient with cellulitis after preparing fresh raw seafood (Koh *et al*., 2008). Thus, these pathogens are suspect causes of zoonosis. This review describes the present status and understanding of SD infection in fish.

1.2.3. Disease Agent

Based on differences in bacteriological characteristics, two subspecies of SD were proposed: *S. dysgalactiae* subspecies *equisimilis* (SDE) and *S. dysgalactiae* subspecies *dysgalactiae* (SDD) (Vandamme *et a*l., 1996). Generally, SD was well known as a pathogen not only in farmed fish, but also in animals and human. Several studies have been reported that SDD or SDE causes mastitis in cattle (Aarestrup and Jensen, 1996), endocarditis in domestic animals, and cardiopulmonary diseases or adenoiditis humans (Efstratiou *et al*., 1994). SD differs in terms of Lancefield serological groupings, heamolytic types, streptokinase activity on different sources of plasminogen, host association, and pathogenicity in host animals. These subspecies of SD are based on phenotypic and genetic differences in heamolysis, whole-cell protein profiles

(Vandamme *et al*., 1996), multilocus enzyme electrophoresis typing, and chromosomal DNA-DNA relatedness (Vieira *et al*., 1998). Strains of SDD react with Lancefield group C or L anti-serum but not with Lancefield group G serum. Streptokinase activity on plasminogen derived from human is not observed. The SDD may be isolated from the respiratory and genital tracts of various animals, but likely not humans (Vandamme *et al*., 1996). SDE strains show beta-hamolysis on the blood agar and react with Lancefield group C or G or L serum. Streptokinase activity on plasminogen derived from human could be observed (Vieira *et al*., 1998). The habitable environment is suspected to the respiratory tracts of host animals. SDE was also isolated from bovine and pigs with carditis (Vandamme *et al*., 1996), and from throat cultures in adult populations (Harrington and Clarridge, 2013).

Table 1.2.1 shows the bacteriological characteristics of SD isolates from fish. Clinical isolates from yellowtail and amberjack showed α-hemolysis on blood agar using several animal erythrocytes (rabbit, cattle, pig, and sheep) at 25°C for 72 h. When incubation of SD on cattle blood agar was prolonged, hemolysis in most isolates changed from alpha to beta (Nomoto *et al*., 2004). Although isolates from Amur sturgeon showed β-hemolysis on crucial carp blood agar at 4°C, no hemolysis was observed at 37°C for more than 7 days (Yang and Li 2009). Nile tilapia isolates showed β-hemolysis at 28°C for 72 h (Netto *et al*., 2011). Lactose utilization was negative in isolates from yellowtail, amberjack, and tilapia. However, Amur sturgeon isolates utilized lactose (Yang and Li 2009). Streptokinase activity in different sources of plasminogen is a key discriminating characteristic between fish and mammalian isolates (Nishiki *et al*., 2010). No fish isolate exhibited streptokinase activity with any source of plasminogen. The biochemical characteristics of α-hemolysis and streptokinase activity of fish isolates from yellowtail and amberjack are important key tests to discriminate from those of typical strains from animals.

Characteristics	**Japanese strains (*n*=10)**	**Brazilian isolate**	**Isolate from human*1**	**Sturgeon isolates**	**ATCC 43078**	**ATCC 35666**
Group antigen (Lancefield group)	C	C	C	nd*2	C	C
Aggregation in growth broth	+				+	+
Haemolysis	α	β	α	β/−*3	α	β
Resitance to 40% bile	−				−	−
Bacitoracin	−				−	−
Growth at 10°C	−			−	−	−
45°C	−			−	−	−
6.5% NaCl	−			−	−	−
PH6.5	−			−	−	−

Voges-Proskauer test	−				−	−
Hydrolysis of hippurate	−	d	−	−	−	−
Esculin	−	d	−	−	−	+
Pyrolidonylamidase	−	−	−		−	−
Galactosidase	−	−	−		−	−
Glucuronidase	+	+	+		+	+
Galactosidase	−	−	−		−	−
Alkaline phosphatase	+	+	+		+	+
Leucine arylamidase	+	+	+		+	+
Arginine hydrolysis	d(3)*4	d	d	+	−	+
Acid from						
Ribose	d(8)	−	d		+	−
Arabinose	−	−	−		−	−
Mannitol	−	−	−		−	−
Sorbitol	−	−	−	−	+	−
Lactose	−	−	−	+	+	−
Trehalose	+	+	+		+	−
Insulin	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−
Sucrose	+				+	+
Amygdaline	+	−	+		+	+
Glycogen	d(5)	d	d		−	+

*1; Data presented by Koh *et al*. (2009) were found to be identical to Japanese strain.
*2; nd (not done).
*3;Hemolytic experiments were conducted at 4°C and 37°C on plates of BHI agar plus 10% blood from crucian carp. β-hemolysis is at 4°C, while negative is at 37°C (Yang and Li, 2009).
*4;d (difference or numbers of positive strains).

Table 1.2.1. Main and differential bacteriological characteristics of fish *Streptococcus dysgalactiae*, *S. dysgalactaie* subsp. *dysgalactiae* ATCC43078, and *S. dysgalactiae* subsp. *equisimilis* ATCC35666. Results are based on compiled data published by Nomoto *et al*. (2004), Koh *et al*.(2009), Yang and Li (2009), and Netto *et al*.(2011)

1.2.4. Diagnostic Methods

1.2.4.1 Clinical Signs and Histopathology

Severe necrosis of the caudal peduncle with abscess and epicarditis is a common symptom in infected farmed amberjack and yellowtail (Figures 1.2.1, 2, 3, and 4). Fish farmers typically find diseased fish in net cages with severe necrosis of the caudal peduncle. When amberjack were experimentally infected with SD through intradermal or intraperitoneal injection, the fish developed micro abscessation and/or granulomatous inflammation of the heart, caudal peduncle (Figure 1.2.3), pectoral and dorsal fins, and olfactory region (Hagiwara *et al*., 2009). These lesions were also observed in amberjack following exposure to SD by oral administration or immersion (Hagiwara *et al*., 2010). SD was isolated from lesions in the caudal peduncle in diseased fish (Nomoto *et al*., 2004). However, the typical subcutaneous abscesses in experimentally infected tilapia cannot be reproduced (Netto *et al*., 2011). Gram-stained smears from necrotic lesion sites may be used to reveal the presence of Gram-positive chain cocci. Although a clinically strong similarity was observed between SD and *L. garvieae* in diseased fish (Nomoto *et al*., 2004), pairs of capsulated cells were observed in Gram-stained smears from lesions in *L. garvieae*-infected fish. While, stained smears from lesions in GCSD infected fish revealed the chains of cells. Selective agar containing Congo red dye was developed to discriminate SD from *L. garvieae* isolated from diseased fish (Abdelsalam *et al*., 2009).

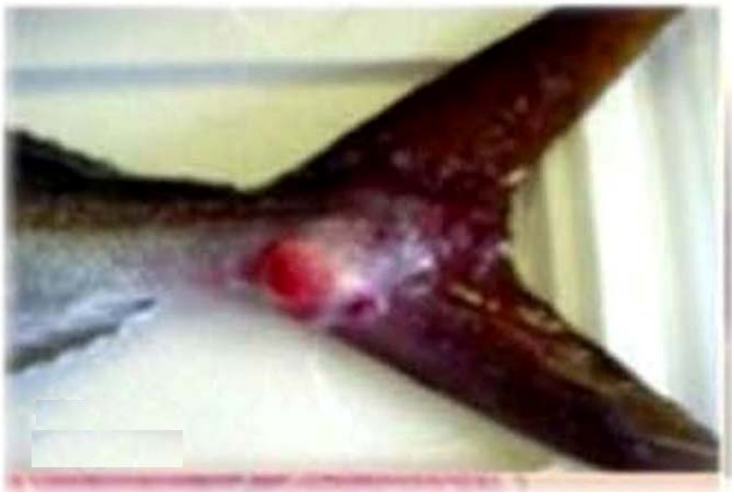

Figure 1.2.1. Severe necrosis with abscess at the caudal peduncle in infected fish

A surface immunogenic protein of fish pathogenic SD (Sd-Sip) that induces an antibody after infection was identified. The recombinant Sd-Sip (rSd-Sip) was applied for the antibody detection by ELISA assay to diagnose SD infection in amberjack (Nishiki *et al*., 2013). Furthermore, the slide agglutinating test using rSd-Sip coated latex beads was developed to detect the agglutinating antibody in SD infected fish at the fish farm side (Nishiki *et al*., 2014).

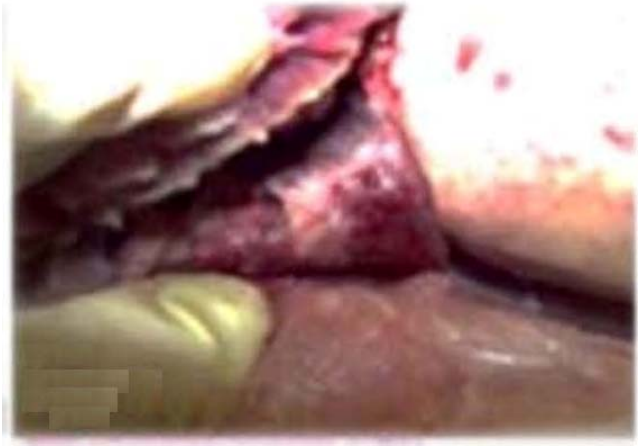

Figure 1.2.2. Severe epicarditis in infected fish

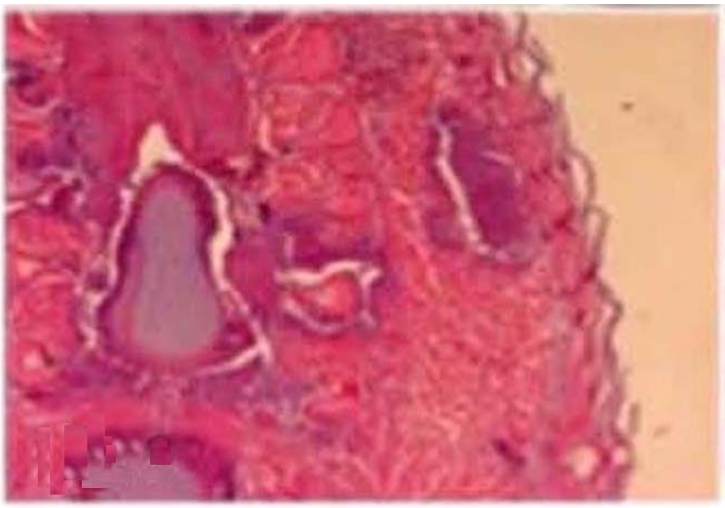

Figure 1.2.3. Histopathology of caudal peduncle in infected fish

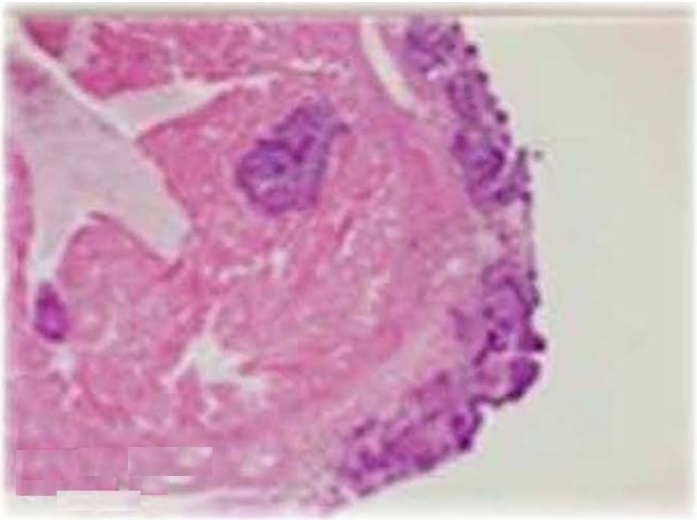

Figure 1.2.4. Histopathology of epicarditis in infected fish

1.2.4.2 PCR

Oligonucleotide primers specifically designed for the 16S-23S rDNA intergenic spacer region of SD we developed for animal isolates (Forsman *et al*., 1997) and were applicable to fish isolates (Nomoto *et al*., 2004). Although a PCR assay using these primers could be used to identify fish SD, this assay could not differentiate fish from animal SD. Sequencing of the *sodA* gene revealed genetic divergence among SD strains isolated from fish and mammals. A PCR assay using the *sodA* gene has been developed to discriminate fish from mammalian isolates (Nomoto *et al*., 2008). A primer set targeting the putative virulence-related serum opacity factor (*SOF*) gene was also designed and used to discriminate fish from mammalian isolates (Nishiki *et al*., 2011). Although the *SOF* gene sequences in different SD isolates from Asian countries, including Japan, Malaysia, and China are identical, different opacification activities are observed. Some types of variations were observed in the upstream region of the gene sequence due to the insertion of IS element into the upstream region of the *SOF* gene (Nishiki *et al*., 2012).

1.2.4.3 Epidemiology

Several epidemiological studies on SD in fish have been conducted. Biased sinusoidal field gel electrophoresis analysis (BSFGE), i.e., macro-restriction profiles, revealed differences in the electrophoretic profiles of fish and animal isolates (Nomoto *et al*., 2006; Nishiki *et al*., 2010). Although various BSFGE profiles were observed in fish isolates, clustering analysis revealed a similarity in fish isolates. DNA-DNA hybridization between fish and mammalian SD ranged from 73.4–82.6%, whereas fish isolates were genetically very close to each other with high DNA-DNA relatedness (95.4%−101.5%). In addition, housekeeping genes such as 16S rDNA, 23S rDNA, *hsp60*, and *sodA* were analyzed to identify differences between fish and mammalian isolates. Fish isolates formed one cluster, distinct from the mammalian isolates. Thus, a clonal expansion of SD strains might occur in fish farms in Japan (Nomoto *et al*., 2006; Nishiki *et al*., 2010). Abdelsalam *et al*. (2010) compared BSFGE typing between fish isolates collected in Japan and other Asian countries. They concluded that isolates from Japan, Taiwan, and China could be grouped into a main cluster at a high similarity level. However, some isolates including those from tilapia were apparently different from those of the main cluster. Netto *et al*. (2011) performed pulsed field gel electrophoresis analysis and reported that isolates from Nile tilapia belonged to a single pulsotype with serotypes. They concluded that the isolates had a restricted geographical (a single outbreak in a farm) and contemporaneous origin.

1.2.5. Control

1.2.5.1 Drug Resistance

Tetracycline resistance was identified in Japanese stains (minimum inhibitory concentration (MIC) value: >25 μg/mL), which carried a resistance gene, *tet*M. The *tet*M gene in fish SD was identical to that in *Enterococcus faecalis* LMG20647 (AJ585078) with 100% similarity. All Japanese strains were sensitive to erythromycin, lincomycin, and ampicillin (Nishiki *et al*., 2009). In particular, MICs of ampicillin against SD strains isolated from yellowtail and amberjack were less than 0.025μg/mL. Some isolates from

Malaysia, China, and Indonesia also showed resistance to tetracycline and carried the *tet*M gene (Abdelsalam *et al*., 2009). Isolates from sturgeon in China showed strong resistance to bacitracin and streptomycin (Yang and Li, 2009).

1.2.5.2 Vaccine

A commercial formalin-inactivated injectable polyvalent vaccine against SD, *L. garvieae*, and *Vibrio anguillarum* serotype J-O-3 (C type) was licensed for use in amberjack cultured in Japan (http://www.kyoritsuseiyaku.co.jp/products/).

1.3. STREPTOCOCCUS INIAE

Terutoyo Yoshida

1.3.1. Synopsis

Steptococcus iniae has been one of the most important disease agents in world aquaculture industries. This pathogen affects a variety of fish species cultured in saltwater and freshwater. Several virulence factors (cell capsule, M-like protein, and streptolysis S) in *S. iniae* that cause disease have been reported. Furthermore, different serotypes in *S. iniae* were suggested based on their reaction to rainbow trout antibodies. Commercial vaccines are available for flounder and tilapia aquaculture in several countries and are given through injection, immersion, or oral adinistration.

1.3.2. Introduction

S. iniae infection occurs in various farmed fishes. *S. iniae* was first isolated from an Amazon freshwater dolphin, *Inia geoffrensis* (Pier and Madin, 1976). Two different pathogens were isolated from tilapia and trout with meningoencephalitis. These isolated bacteria were identified as *S. shiloi* and *S. difficile*（=*S. difficilis*）, respectively (Eldar *et al*., 1994). Later, *S. shiloi* was thought to be a junior synonym of *S. iniae*. (Eldar *et al*., 1995). Recently, *S. iniae* has been isolated from various finfish in freshwater and saltwater. It has also been isolated from humans and may therefore present a zoonotic threat.

S. iniae has been isolated from diseased fish in saltwater, brackish water, and freshwater environments. Estimated damage caused by *S. iniae* infection in the US aquaculture industry in 1997 was around 10 million US dollars (Shoemaker *et al*., 2001). Several cases of invasive *S. iniae* infection concerning human health have been reported (Weinstein *et al*. 1997; Koh *et al*., 2004). This section focuses on *S. iniae* infection in marine fish species.

1.3.3. Disease Agent

S. iniae is a non-typable Lancefield sero-grouping Gram-positive coccus, which forms long chains in broth culture. Colonies of *S. iniae* on blood agar are white and exhibit β-hemolysis (Figure 1.3.1). Activity levels of hemolysis on blood agar are dependent on strains. Some strains isolated from rainbow trout and flounder formed glossy mucoid colonies on blood agar or Todd-Hewitt agar (Figure 1.3.2). Most virulent isolates from

rainbow trout and flounder were encapsulated. The capsule of a mucoid strain is sufficiently developed when visualized by negative staining with Indian black ink (Figure 1.3.3, Yoshida *et al*., 1996). This type of strain may produce large amounts of extracellular polysaccharides. The isolates grow at 37°C and pH 9.6 but not at 45°C, in 40% bile, and in 6.5% NaCl. Acid was produced from aesculin, D-glucose, glycogen, maltose, mannitol, D-mannose, ribose, salicin, starch, and trehalose, but not from amygdalin, D-arabinose, dulcitol, galactose, glycerol, and lactose (Eldar *et al*., 1994). Two serotypes were revealed to exist by the reaction to rainbow trout antibodies. Isolates with 2 serotypes biochemically differed in their ability to respond to arginine dihydrolase and ribose. Genetic evidence of the differences between these serotypes was obtained by a rapid amplified polymorphic DNA technique (Zlotkin *et al*., 2003). Since there was no clear difference in the symptoms of fish infected with different streptococcal pathogens, accurate diagnosis is difficult. In particular, hemolytic *S. iniae* and *S. agalactiae* cause a similar disease in tilapia and sea bream. Table 1.3.1 shows the main bacteriological characteristics of *S. iniae* and *S. agalactiae* based on the report by Evans *et al*. (2006) with a modification.

Tests	***S. iniae***	***S. agalactiae***
Hemolysis on sheep blood agar	+	±
Growth		
at 10°C	+	−
at 45°C	−	−
in 6.5% NaCl	−	−
Production of		
Pyroilidonyl arylamidase (PYR)	+	−
Leucine aminopeptidase (LAP)	+	+
Hydrolysis of		
Hippurate	−	+
Starch	+	−
Lancefield group antigen	ND*	B

ND*; not detection

Table 1.3.1. Main microbiological characteristics between *Streptococcus iniae* and *S. agalactia*e (modification from Evance *et al*., 2006)

1.3.4. Host Range

S. iniae infection occurs in nearly 30 freshwater, saltwater, and euryhaline fish species (Agnew and Barnes, 2007). Infection occurs in cultured and wild species, including economically important species such as tilapia, yellowtail, amberjack, sea bream, trout, and bass.

1.3.5. Diagnostic Methods

1.3.5.1 Pathogenicity

The cell capsule is an important virulence factor for *S. iniae* (=β-hemolytic *Streptococcus* sp.), as it contributes to resistance to opsonophagocytosis (Yoshida *et al*., 1996). Allelic exchange mutagenesis in a virulent *S. iniae* strain produce a capsule-deficient mutant, a decrease in the cell-surface negative charge, buoyancy in liquid culture, elongation of the coccus chain, and virulence in fish (Locke *et al*., 2007a). Virulence of capsular polysaccharide-deleted mutants of *S. iniae* is attenuated (Shutou *et al*., 2007). Pyrosequencing of the *S. iniae* genome revealed an M-like protein (*sim*A) and C5a peptidase homologous genes. Allelic replacement of these genes revealed that the M-like protein plays an important role in *S. iniae* virulence, and the M-like protein mutant might contribute to the development of a live-attenuated vaccine against *S. iniae* infection (Locke *et al*., 2008). Recovery of novel virulent *S. iniae* strain (serotype II) from fish vaccinated with a vaccine of *S. iniae* was observed (Bachrach *et al*., 2001). Two serotypes were revealed to exist by the reaction to rainbow trout antibodies (Zlotkin *et al*., 2003). A serotype II strain can enter and multiply in phagocytes, causing apoptosis (Zlotkin *et al*., 2003). Comparative research of *S. iniae* serotype I and II colonies in infected tissues revealed that type II was responsible for overwhelming septic diseases because the number of live bacteria was larger in type II than in type I (Lahav *et al*., 2004). Loss of streptolysis S production due to allelic exchange mutagenesis led to marked virulence attenuation (Locke *et al*., 2007b).

1.3.5.2 Serotypes

An indirect fluorescent antibody technique based on a monoclonal antibody was developed for rapid detection of *S. iniae* (Klesius *et al*., 2006). Two serological phenotypes exist in Japanese flounder, designated as K^+ and K^-. These serological phenotypes are distinguished by the presence or absence of a cell capsule. The K^- cells agglutinated with anti-K^- and K^+ phenotype sera, whereas K^+ cells agglutinated only with anti-K^- type serum (Kanai *et al*., 2006). The serotypes are differentiated by their reaction to rainbow trout antibodies. These different serotypes can also be differed in a test using arginine dihydrolase and acid production from ribose (Bacharach *et al*., 2001).

1.3.5.3 Molecular Identification

Molecular techniques, such as PCR assays, have been developed for identification and detection of *S. iniae*. The intergenic spacer region of the 16S and 23S ribosomal genes and the lactate oxidase gene were the targets of species-specific primers for identification (Berridge *et al*., 2001; Mata *et al*., 2004).

1.3.5.4 Clinical Signs

Common clinical signs observed in fish infected with in *S. iniae* include erratic swimming, exophthalmia (Figure 1.3.4), lethargy, and massive hemorrhage around the anus. Histopathology revealed that meningitis caused by *S. iniae* resulted in fish mortality. Clinical signs observed in rainbow trout after experimental infection with *S iniae* were

similar to those with *L. garvieae*. However, a histopathological examination revealed notable differences. Meningitis and panophthalmitis were the main lesions caused by *S. iniae*, whereas *L. garvieae* infection caused hyperacute systemic disease (Eldar and Ghittino, 1999).

Figure 1.3.1. β-hemolysis of *S. iniae* on blood agar

Figure 1.3.2. Mucoid type colonies of *S. iniae* isolated from Japanese flounder

Figure 1.3.3. Negative staining by Indian black ink of capsulated *S. iniae* isolated from Japanese flounder

Figure 1.3.4. Infected fish (rainbow trout) showing bilateral exophthalmia

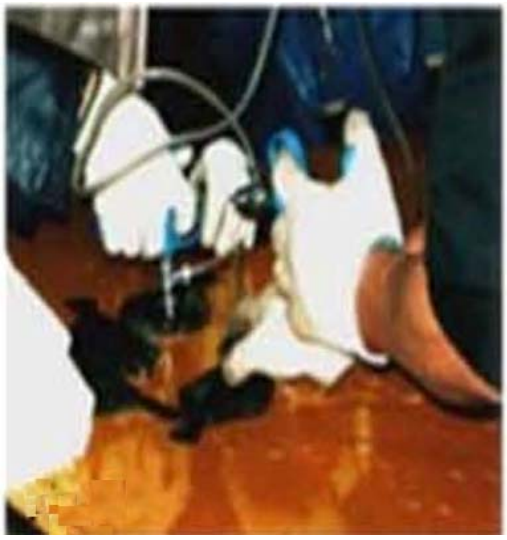

Figure 1.3.5. Injectable vaccine of *S. iniae* for Japanese flounder (Courtesy of Dr. Y. Fukuda, Fisheries Research Division, Oita Prefectural Agriculture, and Fisheries research Center, Oita, Japan)

1.3.5.5 Genomes

The sequence of the polysaccharide capsule synthesis operon related to the virulence of a clinical isolate (*S. iniae* 9117) from the blood of a patient has been deposited in the Genbank under the accession no. AY90444 (Lowe *et al*., 2007).

1.3.6. Control

1.3.6.1 Drug Susceptibility

Antibiotic susceptibility and resistance were investigated in *S. iniae*. Isolates from Korea (n = 65) were susceptible to cefotaxime, erythromycin, ofloxacin, penicillin, tetracycline, and vancomycin (Park *et al*., 2009). Most human isolates were sensitive to anitibiotics such as macrolides, β-lactam, quinolones, and vancomycin (Facklam *et al*., 2005).

1.3.6.2 Vaccination

Formalin-inactivated cells have been used as an effective vaccine for rainbow trout (Bercovier *et al*., 1996) and Japanese flounder (Shutou *et al.*, 2007). Capsular polysaccharides are important protective antigens and an anti-capsular antibody plays a protective role as an opsonin in *S. iniae* infection (Shutou *et al*., 2007). Passive immunization with anti-*S. iniae* serum conferred strong immunity against infection in tilapia (Shelby *et al*., 2002; LaFrentz *et al*., 2011). Therefore, humoral immunity plays an important role in immune protection in tilapia. Immunoproteomic analysis suggested that enolase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase, are likely involved in immune protection against *S. iniae* infection in Nile tilapia (LaFrentz *et al*., 2011). Recently, massive outbreaks of *S. iniae* have occurred in trout farms and a variant of *S. iniae* (serotype II), which had been used for vaccination, was observed in Israel (Bacharach *et al.*, 2001).

Commercial vaccines are available for flounder and tilapia aquaculture in several countries. These vaccines are given through injection, immersion, or oral administration. Figure 1.3.5 shows an injectable vaccine for flounder in Japan. A commercial combined vaccine against *S. iniae* with *S. parauberis* is now available for flounder in Japan (www.maff.go.jp/j/syouan/suisan/suisan_yobo/pdf/26_suiyaku.pdf (in Japanese)).

1.3.6.3 Phage Therapy

The possible therapeutic effects of *S. iniae* phage against *S. iniae* infection were investigated in Japanese flounder. The mortality rate of flounder was significantly reduced with intraperitoneal injection of phages (Matsuoka *et al*., 2007).

1.3.7. Recent Topics

Opportunistic infections of *S. iniae* have been reported in immune-compromised humans (Agnew and Barnes, 2007). Several cases of opportunistic infection were suspected to be associated with injuries during handling of live or infected fish. Symptoms of infection in humans include cellulitis, endocarditis, and meningitis (Lau *et al*., 2003). A potential zoonotic threat due to *S. iniae* infection is becoming clear.

1.4. STREPTOCOCCUS PARAUBERIS

Terutoyo Yoshida

1.4.1. Synopsis

Streptococcus parauberis was originally identified as *S. uberis* type II, an etiological agent of bovine mastitis. *S. parauberis* isolated from diseased turbot was tentatively classified as or *Enterococcus* –like bacterium or *Enterococcus* sp. because *S. parauberis* was closely related to *E. seriolicida*. Doménech *et al*. (1996) reported *S. parauberis* as a fish pathogen in turbot *Scophthalmus maximus* in Spain. Recently, *S. parauberis* has also been isolated from flounder *Paralichthys olivaceus* in Japan and Korea. *S. parauberis* isolated from flounders have been recognized as serotypes I and II, and vaccine application for *S. parauberis* infection has been investigated.

1.4.2. Introduction

S. parauberis isolated from diseased turbot was tentatively classified as *Enterococcus* sp. because *S. parauberis* was closely related to *E. seriolicida* in Spain (Toranzo *et al*., 1994 and1995; Doménech *et al*., 1996). *S. parauberis* was originally identified as *S. uberis* type II, a genotypically distinct group of isolates of *S. uberis* type I, an etiological agent of bovine mastitis (Williams and Collins 1990). *S. parauberis* infections in turbot aquacultures have been prevalent in Mediterranean countries (Doménech *et al*., 1996; Ramos *et a*l., 2004).

In streptococcosis, *Streptococcus iniae* infection was the most serious Gram-positive bacterial disease in flounder in Japan and Korea until the emergence of *S. parauberis*. Although *Lactococcus garvieae* and *S. iniae* infections have been prevalent in Japan, emerging *S. parauberis* infection is becoming one of the most important fish bacterial

diseases in flounder aquaculture. Recently, a vaccine consisting of formalin-killed cells with toxoids was found to be effective. A commercial vaccine against *S. parauberis* with *S. iniae* is now available for flounder in Japan (www.maff.go.jp/j/syouan/suisan/suisan_yobo/pdf/26_suiyaku.pdf (in Japanese)).

1.4.3. Disease Agent

Whitish colonies of turbot isolates with slight α-hemolysis grow on Columbia agar supplemented with 5% defibrinated sheep blood. Turbot isolates do not grow at 4°C, 45°C, pH 9.6, and in 6.5% NaCl. Acid was produced from ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, maltose, and lactose. Positive reactions were observed for arginine, pyrroidnyl arylamidase and in a Voges-Proskauer test. Variable reactions in hydrolysis of hippurate are observed (Doménech *et al*., 1996). *S. parauberis* isolated from the farmed flounder showed variable reactions in acid production from lactose (Han *et al*., 2011). Significant differences in biochemical characteristics using Voges-Proskaue test, pyrrodonyl arylamidase asn hemolysis was observed between *S. parauberis* and *S. iniae* isolates from olive flounder (Nho *et al*., 2009). *S. parauberis* with both serotypes possessed a capsular layer around the cell surface when the bacterial cells were pretreated with serotype-specific rabbit antiserum (Han *et al*., 2011).

1.4.3.1 Serotypes

Regardless of isolation year, turbot isolates of *S. parauberis* in Spain showed the same phenotypic and serologically homogeneous group, which has facilitated to the development of a vaccine (Tranzo *et al*., 1995). However, two serotypes were revealed in agglutination tests using rabbit antisera with isolates from flounder in Japan (Kanai *et al*., 2009) and Korea (Han *et al*., 2011). Capsular polysaccharide antigens might act to differentiate the serotypes of *S. parauberis* in olive flounder. Antisera raised against *S. parauberis* types I and II isolated from flounder could not be agglutinated with the *S. parauberis* ATCC13386 cells isolated from mammal (Kanai et al., 2009). They suggested that the reference strains ATCC 13386 strain could be different serotypes from flounder isolates. Serotype I strains are the more dominant isolates in olive founder (Han *et al*., 2011). However, these serotypes could not be differentiated from each other based on serotype-specific biochemical characteristics and 16S-23S rRNA intergenic spacer region (ISR) sequences (Han *et al*., 2011). Immunoblot analysis of *S. parauberis* whole-cell lysates collected from olive flounder using a chicken anti-S. *parauberis* IgY antibody revealed 3 distinct antigenic profiles (Nho *et al*., 2009).

1.4.3.2 Pathogenicity

Serotype I strains are associated with greater mortality than serotype II strains. The LD 50 values of serotype I strains were 1.0×10^7 and 1.0×10^8 CFU fish^{-1}, whereas those of serotype II strains were more than 1.0×10^9 CFU fish^{-1}. Both two serotypes possessed cell capsule layer around the cell surface (Han *et al*., 2011). The cell capsule of *S. parauberis* plays an important role in resistance to sera and phagocytosis in olive founder (Hwang *et al*., 2008). However, no specific characteristics between the two serotypes were observed (Han *et al*., 2011). *S. parauberis* could exist in the viable but non-culturable state (VNBC), and dormant cells of a turbot isolate could maintain their infectivity and

pathogenicity (Curras *et al*., 2002). Infection routes of *S. parauberis* were investigated to reproduce experimental infection tests as a naturally infected fish. Subcutaneous injection at the base of the dorsal fin or outside the preopercular bone is recommended for experimental challenge with *S. parauberis*. Similar histopathological signs were observed between artificially and naturally infected diseased flounder (Mori *et al*., 2010).

1.4.3.3 Genome Analysis

The complete genome sequence of serotype I *S. parauberis* (KCTC11537BP) isolated from diseased olive flounder in Korea was determined and deposited as an accession no. CP002471 (Nho *et al*., 2011). The *S. parauberis* genome contains 2,143,887 bp with 1,868 predicted coding sequences and 35.6% G+C content. The KCTC11537BP strain is evolutionarily and closely related to *S. uberis*, according to the whole-genome dot plot analysis and phylogenetic analysis of a 60-kDa chaperonin-encoding gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-encoding gene sequences (Nho *et al*., 2011).

1.4.4. Diagnostic Methods

1.4.4.1 Clinical Signs

Cases of turbot mortality occur throughout the year, although the clinical signs are more severe in summer with slightly higher mortality rate. Typical external clinical signs in turbot were hemorrhage in the anal and pectoral fins, petechiae on the abdomen, exophthalmos, and pus in the eye (Doménech *et al*., 1996). Gross pathology included gill necrosis and severe hemorrhage in the liver and the trunk muscle was observed in Japanese flounder (Kanai *et al*., 2009). The experimentally challenged fish showed severe hemorrhages at various sites with ascetic fluid in the peritoneal cavities (Kim *et al*., 2006). Figure 1.4.1 and Figure 1.4.2 showed infected flounder showing severe hemorrhages of liver with ascetic fluid and gill necrosis.

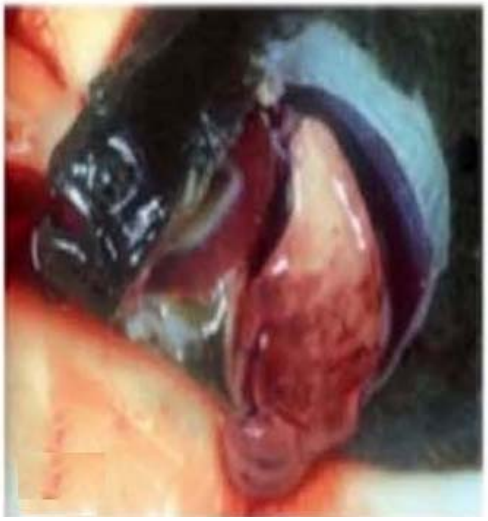

Figure 1.4.1. Infected flounder showing severe hemorrhages of liver associated with ascites (Courtesy of Dr. Y. Fukuda, Fisheries Research Division, Oita Prefectural Agriculture, and Fisheries research Center, Oita, Japan)

Figure 1.4.2. Infected flounder showing severe gill necrosis (Courtesy of Dr. Y. Fukuda, Fisheries Research Division, Oita Prefectural Agriculture, and Fisheries research Center, Oita, Japan)

1.4.4.2 PCR for Diagnosis

PCR assays targeting the 16S rRNA, 23S rRNA, or 16S-23S rRNA ISR were developed to identify and differentiate *S. parauberis* from *S. uberis* isolates from bovine mastitis (Hassan *et al*., 2001). The PCR assay targeting the species-specific *sod*A and *cpn*60 was developed to allow a rapid and reliable PCR mediated identification of *S. parauberis* and *S. uberis* (Alber *et al*., 2004). A multiplex PCR assay was developed to detect pathogens associated with streptococcosis caused by *S. parauberis*, *S. iniae*, *S. difficilis*, and *Lactococcus garvieae* in infected fish (Mata *et al*., 2004).

1.4.5. Control

1.4.5.1 Drug Resistance

Erythromycin and tetracycline resistance were observed in isolates from flounder (Kanai *et al*., 2009). The *tet*M and *tet*S genes mediated tetracycline resistance in *S. parauberis* isolates from olive flounder, and the *erm*B gene mediated high-level resistance to erythromycin (Park *et al*., 2009). Resistance strains of serotype I possess both *tet*S and *erm*B genes. The *tet*S gene is encoded in the plasmid, whereas the *erm*B gene resides on the chromosomal DNA (Meng *et al*., 2009a). Tetracycline resistance strains of serotype II possess the *tet*M gene, encoded in the Tn916-related element of the chromosomal DNA (Meng *et al*., 2009b).

1.4.5.2 Vaccine and Probiotics

A toxoid-enriched whole-cell bacterin against *S. parauberis* (=*Enterococcus* sp.)

infection was effective in cultured turbot. High degrees of protection lasted for at least a year (Toranze *et al*., 1995). In vaccine trials in flounder, formalin-killed cells of both serotypes (I and II) were effective against challenges with a homologous serotype strain, whereas the vaccine efficacy against the heterologous serotype was not consistent in the mortality and reservoir rates (Mori *et al*., 2012). A combined formalin-killed serotype I and II vaccine is licensed for use in flounder in Japan.

1.4.5.3 Probiotics

Probiotics including *Lactobacillus plantarum*, *L. acidophilus*, *L. brevis*, *Bacillus subtilis,* and *Saccharomyces cerevisiae* and herbal mixture supplementation diet enhance growth, blood composition, and nonspecific immune response to experimental infection with *S. parauberis* in flounder (Harikrishnan *et al*., 2011).

The *L. sakei* BK19 supplemented diet (10^8 cells g^{-1}) fed to grouper, *Epinephelus bruneus* for two weeks reduced the mortality in the experimental challenge with *S. parauberis* as compared to the non-supplementated group. The immune response in probiotics fed group showed significantly increased phagocytic and peroxidase activities (Harikrishnan *et al*., 2010).

1.4.6. Recent Topics

S. parauberis was recovered from a spoiled vacuum-packaged refrigerated seafood product. Isolates were identified by 16S rRNA gene sequencing and characterized using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The MALDI-TOF MS allowed rapid and direct identification of *S. parauberis* (Fernandez-No *et al*., 2012).

Glossary

MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

2. NOCARDIOSIS

Masahiro Sakai

2.1. Synopsis

Nocardiosis caused by the bacterium, *Nocardia seriolae,* has made serious damage in Japanese mariculture. Typical disease signs appear as nodules in gills, spleen, kidney and liver. Although the progression of the disease is chronic and slow, the mortality rate may reach 50% or more. As this bacterium is believed to be intracellular, it is difficult to effectively treat with drug administration. Therefore, the development of effective vaccines against nocardiosis is necessary.

2.2. Introduction

Nocardiosis in fish was first described by Rucker (1949) as *Streptomyces salmonicida*

infection in sockeye salmon (*Oncorhynchus nerka*). This bacterium was identified as *Nocardia salmonicida*, based on the presence of meso-diaminopimelic acid, arabinose and galactose in the whole organism hydrolysates. The outbreak of nocardiosis was first reported from cultured marine fishes such as yellowtail (*Seriola quinqueradiata*) in Mie Prefecture, Japan, in 1967 (Kariya et al, 1968) and then it spread to fish farms in the western districts of Japan. This disease is characterized by the formation of abscesses in the epidermis and of tubercles in gills, kidneys, and spleens (Kariya et al, 1968; Kusuda and Taki, 1973). In 1968, the causative organism was isolated and proposed as a new species, "*Nocardia kampachi*". After that, Kudo et al. (1988), on the basis of deoxyribonucleic acid (DNA)-DNA hybridization and mycolic and fatty acid profiles in addition to physiological and biochemical characteristics, proposed a new species, *Nocardia seriolae*.

At present, this infection is feared as the greatest damage causing disease in yellowtail and amberjack aquaculture. Outbreaks of nocardiosis in the farms usually initiate in September or October and terminate in November. These results suggest that infection of *N. seriolae* in yellowtail begins between July and August (Itano et al, 2008).

2.3. Characteristics of the Disease

The internal pathology of nocardiosis is easily confused with other white-spot-forming diseases, such as mycobacteriosis (fish tuberculosis) and photobacteriosis (formerly Pasteurella or pseudo-tuberculosis), especially if mixed infections exist. The white-yellow granulomas are usually 1-2 mm in size. The spots are most obvious in the spleen, kidney and liver but can be found in any tissue. Fish mount a significant immune reaction and exhibit hard black spots (melano-macrophage accumulations) in place of the white spots in the liver and adipose tissues. Brown-black crusty plaques often develop on the dorsal inner surface of the swim bladder (Sheppard, 2005). Typical disease signs include nodules in gills, spleen, kidney and liver with or without multiple skin ulcers/abscesses (Austin and Austin, 2007). Histo-pathologically, the observed lesions are typical granulomas (Egusa, 1983). Cornwell et al. (2011), reported that fish infected with *N. seriolae* had necrotic external lesions on the caudal peduncles, the lower jaw, the flank near the base of the left pectoral fin, and the dorsal skull. Internally, infected fish has multiple raised white foci on the posterior kidney.

2.4. Disease Agent

The causative agent of Nocardiosis is *Nocardia seriolae*. This bacterium is a Gram-positive, acid-fast, aerobic, non-motile, pleomorphic rod-shaped bacterium (Figure 2.1). The morphology of *N. seriolae* varies, but cells are generally filamentous, branched or beaded. The bacterium is acid-fast and can grow on a variety of media containing carbon and nitrogen sources. The isolates of *N. seriolae* contain meso-diaminopimelic acid, arabinose and galactose, suggesting chemotype IVA. Iso- and anteiso-branched acids have not been detected. The total number of carbon atoms in the mycolic acids is from 44 to 58. The predominant isoprenoid quinone is tetrahydrogenated menaquinone with eight isoprene units. The G + C ratio of the DNA is 66.8-67.4 mol % (Austin and Austin, 2007).

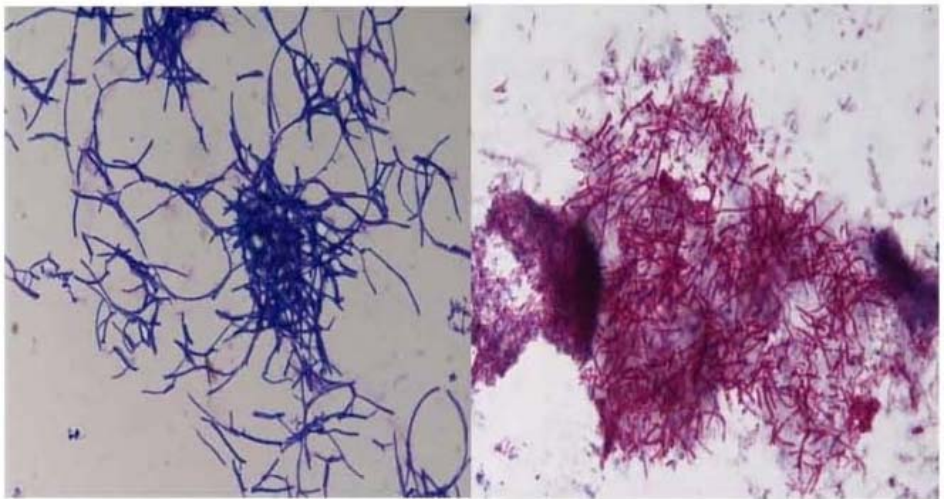

Figure 2.1. Gram-stain from infected fish showing typical branching Gram-positive hyphae indicative of nocardial infections (X1000). **b:** Ziehl-Neelsen stain from infected fish showing typical acid-fast branching hyphae (X1000). (Photos courtesy of Prof. T. Yoshida and Dr. TFIM Ismail)

2.5. Diagnosis

Figure 2.2 shows the typical fish nocardiosis signs with multiple focal or diffuse skin ulcers (upper) and spleen (lower). The typical external symptoms are: thin fish, skin nodules (focal, multifocal or coalescing), skin ulceration, opercular erosion and irregularly-shaped fleshy white masses at the base of the gill filaments (Sheppard, 2005). The internal pathology of nocardiosis is easily confused with other white-spot-forming diseases, such as mycobacteriosis and photobacteriosis. The white-yellow granulomata are usually 1-2 mm in size. The spots are most obvious in the spleen, kidney and liver but can be found in any tissue.

N. seriolae as causative agent can be isolated on brain heart infusion agar (BHIA), tryptone soya agar (TSA) and nutrient agar (NA), with optimum growth at 20–30°C temperature (Kusuda and Taki, 1973). It produces flat, wrinkled colonies after 10 days at 25°C.

The molecular method using polymerase chain reaction (PCR) and Loop-mediated isothermal amplification (LAMP) has been developed and used for detection of *N. seriolae* infection in fish (Kono et al, 2002; Miyoshi and Suzuki, 2003; Itano et al, 2006). Itano et al. (Itano et al, 2006) reported that the detection of *N. seriola* using LAMP was found to be more sensitive than that by PCR.

Figure 2.2. Typical disease signs of nocardiosis include multiple focal or diffuse skin ulcers (upper), and spleen (lower left). (Photos courtesy of Prof. T. Yoshida and Dr. TFIM Ismail)

2.6. Control

The use of chemotherapeutics for the treatment of nocardiosis has been reported (Yasumoto and Yasunaga, 1986; Hatai et al, 1984). Yasumoto and Yasunaga (Yasunaga, 1986) also reported that the administration of erythromycin (EM) or spiramycin (SPM) was not effective in treating yellowtails that were naturally infected with nocardiosis. However, Hatai et al. (Hatai et al, 1984) reported that EM was effective for the treatment of yellowtails that were artificially infected with *N. kampachi*, although SPM, chloramphenicol (CP), oxolinic acid (OX), and sulfamonomethoxine (SMM) were not. Itano and Kawakami (Itano and Kawakami, 2002) reported that several *N. seriolae* strains show resistant to EM and SPM. Due to emergence of these drug-resistant bacteria it is very difficult to treat the disease.

Vaccination may be the most effective method to prevent the disease. However, to date, no effective vaccine against this infection has been developed. Shimahara *et al.* (Shimahara et al, 2005) reported that no protective effects resulted from immunization with formalin-killed *N. seriolae* cells or formalin-killed cells with Freund's incomplete adjuvant, even though antibody levels increased. Itano et al. (2006) attempted to use phylogenetic relatedness and antigenic cross-reactivity to identify vaccine candidates against *N. seriolae*. The authors used environmental *Nocardia* species and evaluated the ability of these isolates (*N. soli* and *N. fluminea*) to induce protective immunity against *N.*

seriolae in injected yellowtail. Unfortunately, the isolates provided minimal protection to a challenge with virulent *N. seriolae*.

2.7. Conclusion

In Japanese marculture, nocardiosis is a disease most difficult to control. No effective drug or a vaccine has so far been developed. Therefore, the only prophylaxis is to remove the infected fish, and not to give stress to fish. In the future, development of effective vaccine against this disease is much anticipated.

Glossary

BHIA: Brain heart infusion agar,

PCR: Polymerase chain reaction,

LAMP: Loop-mediated isothermal amplification,

3. MYCOBACTERIAL DISEASE

Kim D. Thompson and Alexandra Adams

3.1. Synopsis

Fish mycobacteriosis (or fish tuberculosis), caused by *Mycobacterium* spp., is a progressive disease affecting a wide range of wild and cultured marine and freshwater fish species. The economical losses experienced by the aquaculture industry due to mycobacteriosis, the lack of effective treatment regimes and the zoonotic nature of the bacteria involved, highlight the need for rapid methods to detect and identify the bacterial species associated with disease.

Detection and identification of the mycobacteria is traditionally based on histopathology, culture, and biochemical properties, although these do not offer the sensitivity or specificity of nucleic acid-based amplification methods. As with other bacterial pathogens, DNA-based techniques have revolutionised the identification and classification of the mycobacteria. *M. marinum*, *M. fortuitum* and *M. chelonae* are the species most predominantly associated with mycobacteriosis. However, the recent use of genotyping-based techniques has resulted in an increase in the identification of a number of new *Mycobacterium* spp. associated with mycobacteriosis, and highlighted problems with polymerase chain reaction (PCR) amplification due to cross-reactions of species-specific primers with closely related mycobacteria spp.

3.2. Introduction

Mycobacteriosis, caused by non-tuberculosis mycobacteria (or atypical mycobacteria), has been reported in a wide range of freshwater and marine fish species, and can result in significant economic losses to the aquaculture industry. Three species of *Mycobacterium*, *M. marinum*, *M. fortuitum* and *M. chelonae,* have been cited as the main species involved in these infections, although various other non-tuberculous mycobacteria, including a

number of new species, have also been associated with disease outbreaks in fish (Gauthier and Rhodes, 2009).

The occurrence of clinical outbreaks is associated with water temperature, over-crowding, poor nutrition and poor environmental conditions, and infection is believed to occur by both vertical and horizontal transmission.

Many of the mycobacteria spp. that infect fish also have the potential to infect humans, especially immunocompetent or immunosuppressed individuals. Phylogenetic studies have shown *M. marinum* to be closely related to *M. ulcerans* and *M. tuberculosis,* which shares many of their virulence factors and pathological traits. It is therefore used as a model organism to study the pathogenesis of *M. tuberculosis* in poikilotherm model species such as zebrafish (*Danio rerio*), and medaka (*Oryzias latipes*).

Recent research has focused on more accurate methods of identifying and speciating the mycobacteria spp. involved in mycobacteriosis and the development of vaccines.

3.3. Disease Agent (Characteristics, Genome Size, Serological Classification, Molecular Classification, Pathogenesis)

Mycobacterium spp. belong to the family *Mycobacteriaceae* of the actinomycetes (Pitulle *et al.*, 1992). They are aerobic, non-motile, acid fast, Gram-positive bacteria (although this can be difficult to confirm), with a curved or rod shaped morphology (0.2-0.6×1.0-10 μm). Classification of species within the *Mycobacterium* genus is complex, and current classification is based on a polyphasic approach using phenotyping, chemo-taxonomy and molecular analysis (Adékambi and Drancourt 2004; Wallace *et al.*, 2005). Mycobacteria have a diverse range of phenotypic characteristics (Smole *et al.*, 2002), and are divided into two groups based on their pathogenicity i.e. tuberculosis and non-tuberculosis (or atypical) mycobacteria (Eisenstadt and Hall, 1995). Non-tuberculosis mycobacteria are characterised based on their growth (slow or rapid grow), their colony colour (white/cream to yellow/orange) and the biochemical composition of their cell walls. *Mycobacteria* have a chemotype IV cell wall composition containing mycolic acid. Phenotypic methods can have limited ability to discriminate between mycobacteria spp. due to interspecies homogeneity, intraspecies variability, and the existence of unclassified species (Springer *et al.*, 1996; Tortoli *et al.*, 2001). On the other hand, molecular taxonomy has had a significant impact on the classification of the genus *Mycobacterium* (Tortoli, 2003). The 16S ribosomal RNA (rRNA) sequence can differentiate between slow and rapidly growing species; the majority of slowly growing mycobacteria contain a long helix 18 at position 430 - 500 (Tortoli, 2003), and the majority of rapidly growing mycobacteria contain two copies of the 16S rRNA gene (Helguera-Repetto *et al.*, 2004).

Although PCR has been used for the rapid identification of *Mycobacterium* spp, there are problems with closely related species within the genus cross reacting with species-specific primers and commercially available probes. Additional methods are therefore required to identify mycobacteria to species level, such as PCR-restriction enzyme pattern analysis (PRA) and sequence analysis. Nucleic acid sequencing is frequently used to identify mycobacteria to species level, i.e. fragments or an entire gene,

amplified by PCR, are sequenced and compared to known sequences within available databases e.g. GenBank. Regions in the *Mycobacterium* genome used for this include the 16S rRNA gene (Kirschner *et al.*, 1993), the heat shock protein 65 gene (*hsp65*) (Ringuet *et al.*, 1999), the internal transcribed spacer 1 (ITS1) (Roth *et al.*, 1998), *dnaJ* (Yamada-Noda *et al.*, 2007), and the ß subunit of RNA polymerase gene (*rpoB*) (Kim *et al.*, 1999). The presence of identical/highly similar 16S rRNA sequences between species has limits its use as a target for species differentiation (Clarridge, 2004). As a result of the sequence variability within the 16S-23S spacer region, several ITS1 sequence based assays have been developed as an alternative region for the identification of mycobacteria (Pourahmad, 2007).

The *M. marinum* genome is composed of a single circular chromosome of 6,636,827 bp, with 5424 protein-coding sequences (CDS), 65 pseudogenes, 46 tRNA genes, a single rRNA operon and a 23 kb mercury-resistance plasmid (pMM23) (Stinear *et al* 2008). It also contains an ESX-1 secretion system essential for mycobacterial pathogenesis (Lewis & Chinabut, 2011).

As well as *M. marinum*, *M. fortuitum* and *M. chelonae,* several other mycobacteria spp. have been associated with mycobacteriosis in fish. A selection of these include *M. abscessus*, *M. avium, M. barombii, M. chesapeaki*, *M. gordonae*, *M. haemophilum M. lentiflavum-like, M. montefiorense; M. montefiorense*-like', *M. neoaurum*, *M. pregrinum/septicum*, *M. pseudoshottsii, M. salmoniphilum, M. scrofulaceum*, *M. shottsii, M. simiae, M. triplex-like* and *M. szulgai* (Gauthier & Rhodes, 2009; Pourahmad *et al* 2009; Zerihun *et al* 2011). The susceptibility of fish to *M. ulcerans* is still unclear (Gauthier & Rhodes, 2009; Mosi *et al,* 2012).

Mycobacteria are facultative intracellular bacterial pathogens (Pasnik *et al.*, 2003), able to survive and replicate in host macrophages by preventing phagosome maturation (Rybniker *et al.*, 2003; Solomon *et al.*, 2003). They are also zoonotic, and have been associated with tuberculoid infections in people using public swimming pools and are a hazard for people involved with aquaculture and aquarium-related industries and hobbies. The pathogen enters through cuts or broken skin, resulting in the formation of skin lesions on hands or extremities 3 weeks to 9 months after contact with the pathogen.

3.4. Diagnostic Methods (Clinical Signs, Gross Pathology, Histopathology, Diagnosis: PCR, Antibody)

Mycobacteriosis is a chronic disease, sometimes taking months or years for clinical signs to appear (Hedrick *et al.*, 1987; Knibb *et al.*, 1993). The severity of the disease can vary dramatically, with only a few fish dying from chronic infections or high levels of mortality during severe outbreaks (Whipps *et al.*, 2003). Clinical signs of mycobacteriosis tend to be non-specific, but may include emaciation, anorexia, exophthalmia, ascites, pigmentation changes and dermal ulcerations. On post-mortem examination, grey-to-white granulomas are evident (e.g. especially in anterior kidney, spleen, mesenteries and liver). Identification of acid fast bacteria in lesions by Ziehl-Neelsen (Figure 3.1) is, however, both non-specific and not especially sensitive.

Culture is traditionally considered the gold standard for definitive diagnosis of *Mycobacterium,* using specialised media such as Lowenstein-Jensen, Petragnani, Sautons, Middlebrook 7H10, and Dorset egg media at 20 to 30^{o}C. It can take between 2 to 30 days before colonies appear and often cultures become overgrown with contaminating bacteria. Initial characterisation of bacteria is based on growth characteristics (e.g. pigment production, growth rate, and gross and microscopic colony morphologies) and biochemical activity.

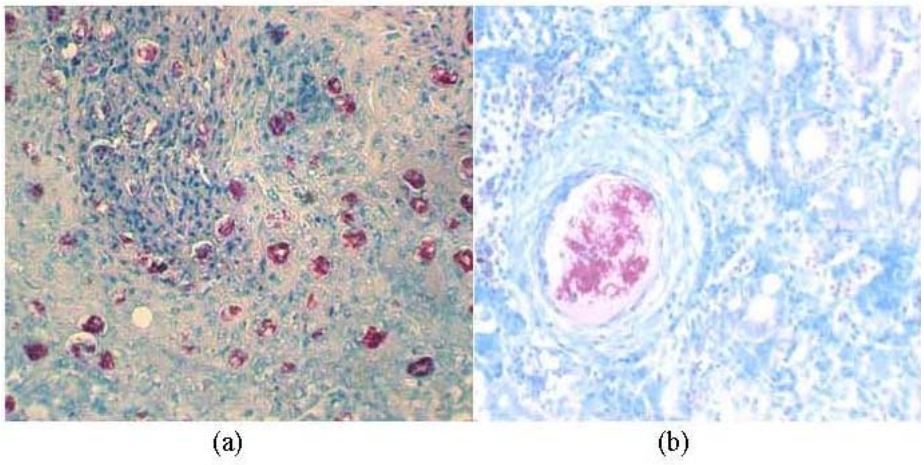

Figure 3.1. Representative Ziehl-Neelsen-stained sections of fish tissues showing granumatous reactions. (a) Spleen tissue of a cichlid following natural infection with *M. stomatepiae* (x200) (b) Kidney tissue of a rosy barb naturally infected with *Mycobacterium* sp. (x·400) (modified from Pourahmad *et al*, 2009)

Molecular detection methods based on nucleic acid amplification have great potential for rapid diagnosis of mycobacteria (i.e. PCR amplification of species-specific sequences (Devallois *et al.*, 1996), amplification and restriction enzyme analysis (Telenti *et al.*, 1993; Talaat *et al.*, 1997), hybridization with species-specific DNA probes (Alcaide *et al.*, 2000), PCR followed by reverse cross blot hybridization (Puttinaowarat *et al.*, 2002) and DNA sequencing (Kirschner *et al.*, 1993; Swanson *et al.,* 1996).

Monoclonal antibodies have been produced against various mycobacteria for use in immunohistochemistry, ELISA and Western blotting (Verstijnen *et al.*, 1991, Adams *et al.*, 1995 and 1996; Blackwell *et al.*, 2001). However, false-positive results from cross-reaction with environmental mycobacteria can be an issue (Ramachandran and Paramasivan, 2003).

3.5. Control (Prevention, Chemotherapy, Vaccine)

Mycobacterium infections in fish can be very difficult to treat, especially since no commercial vaccines are available for mycobacteriosis. Treatment with antibiotics can be time-consuming, expensive, and the bacterium is often resistant to the antibiotics commonly used in aquaculture. Also, there are no authorized antibiotics currently available to treat *Mycobacterium* infections in fish (Stoffregen *et al.*, 1996). It is also unclear if the use of antibiotics eliminates the bacterium or if fish become asymptomatic

carriers as a result. In light of this, the best approach for controlling mycobacteriosis is through good bio-security. The most effective course is to destroy infected stock, disinfect the system, and restock with *Mycobacterium*-free fish.

Mycobacteria are more resistant to disinfection than other bacteria; their waxy cell wall protects them from many of the commonly used disinfectants. Ethyl alcohol (50% and 70%), benzyl-4-chlorophenol/phenylphenol (1%), and sodium chlorite (mixed as 1:5:1 or 1:18:1[base: water: activator]) have been shown to be good mycobacteriocidal agents, killing all detectable *M. marinum* within 1 min of contact (Mainous and Smith, 2005). Lysol®, Roccal® and Virkon® are three commercially available mycobacteriocidal agent used in aquaculture.

Stringent quarantine procedures are required prior to adding new fish to a system. Quarantine times of 30 days are required to allow clinical signs of mycobacteriosis to develop, and additional confirmation that fish are *Mycobacterium*-free may be necessary, and the sensitivity of molecular methods may be necessary to confirm the fish's *Mycobacterium* status. It may not be possible to kill expensive ornamental fish to screen for mycobacteria however, and there are currently no non-lethal sampling methods available for screening fish for mycobacteria.

3.6. Recent Topics

There has been increased interest in the development of improved methods to detect and differentiate the *Mycobacterium* spp infecting fish. This, in part, is due to the increased incidence of mycobacteriosis in aquaculture systems, not only for food and ornamental fish, but also for zebra fish used in clinical research to develop specific pathogen-free colonies. Recent developments for detection include FRET assays (Salati *et al.*, 2009) and sensitive real time PCR methods (Lloyd *et al.* 2009, Parikka *et al.* 2012), and for differentiating *Mycobacterium* spp., polygenic sequencing (Pourahmad, 2007; Kurokawa *et al*. 2012) and a MALDI Biotyping system (Kurokawa *et al*. 2012). Attempts to develop an effective vaccine for mycobacteriosis also continues (Kato *et al.* 2010, 2011).

Glossary

PCR: Polymerase chain reaction,

FRET assays: Fluorescence/förster resonance energy transfer

4. PASTEURELLOSIS

Tae-Sung Jung and Takashi Aoki

4.1. Synopsis

Pasteurellosis, caused by *Photobacterium damselae* subspecies *piscicida,* appeared in 1963 and has since been causing problems to the aquaculture industry. It is especially known to infect various fish species including yellowtail in Japan and sea bass and sea bream in the Mediterranean region. Pasteurellosis can be controlled using antibiotics at the initial stages of infection but have also led to the emergence of antibiotic resistant

strains. It was found to be pathogenic to mammals including humans and recently have been reported to infect fish in colder and tropical regions. It is therefore important to understand the bacterial pathogenesis including bacteria virulence factors to develop highly effective vaccines. Here, the characteristics of the disease agent, the methods for diagnosis, prevention and control, and recent information about this disease will be discussed.

4.2. Introduction

Pasteurellosis or pseudotuberculosis in Japan was first observed in wild white perch (*Morone americanus*) and striped bass (*M. saxatilis*) in 1963 at Chesapeake Bay, USA (Snieszko et al, 1964). It has since been observed in cultured yellowtail (*Seriola quinqueradiata*) in Japan and was confirmed to be caused by *Photobacterium piscicicda* (Kubota et al, 1970). It was later reported in cultured gilthead sea bream (*Sparus aurata*) and in sea bream and sea bass in Europe, mainly in Spain (Toranzo et al, 1991). The biochemical and serological characteristics of *P. piscicida* isolates obtained from the USA, Japan and Europe are highly similar. It was later renamed as *P. damselae* subspecies *piscicida*, after it was found that there is high DNA-DNA relatedness (80%) between *P. piscicida* (NCIMB2058) and *P. damselae* subsp. *damselae* (Gauthier et al, 1995). The name *P. damselae* subsp. *piscicida* is currently more widely used.

4.3. Characteristics of the Disease Agent

4.3.1 Characteristics

P. damselae subsp. *piscicida* is a non-motile, Gram-negative, rod-shaped bacterium, 0.5 x l.6 μm in size. It exhibits a bipolar staining, oxidase- and catalase- positive, fermentative, does not produce gas from glucose, and halophilic (Magariños et al, 1992). It differs biochemically from other common fish pathogens such as *Vibrio anguiIlarum* and *Aeromonas salmonicida* subsp. *Salmonicida,* as well as from other Pasteurella spp. (Table 4.1). Compared with *Pasteurella* spp., *P. damselae* subsp. *piscicida* does not grow at 37^{o}C, is halophilic and does not produce nitrate. *P. damselae* subsp. *piscicida* also differs from *P. damselae* subsp. *damselae* in motility and biochemical characteristics (Thyssen et al, 1998).

4.3.2 Genome Size

There is no available report with regards to the whole genomic sequencing data, but several genes such as plasmid (Zhao and Aoki, 1992), 16S rRNA and bacterial capsule were partially sequenced especially for diagnosis and to explore antibiotic resistance and virulence related genes (Osorio et al, 2008). Random sequencing of the genomic DNA of *P. damselae* subsp. *Piscicida* was conducted and 930 contigs were assembled (Naka et al, 2005). Recently, the whole genome shotgun sequence was submitted to the NCBI database but is not published until now (Balado, Lemos and Osorio, 2012: NCBI nucleotide database).

Gram stain	-	Phenylalanine deamination	-
Bipolar staining	+	Gluconate utilizati	-
Cell morphology	Short rods	D-Tartrate	-
Motility	-	Gelatinase	-
Growth on nutrient agar	+	Caseinase	-
In nutnent broth	+	Lipase (Tween 80)	+
In peptone water	+	Phospholipase	+
On heart-infusion agar	+	Amylase	-
On BHIagar	+	Haemolysis; Sheep erythrocytes	-
On SS agar	-	Haemolysis; Salmon erythrocytes	-
On MacConkey agar	-	Acid production from: Glucose	+
On Endo agar	-	MannosE	+
Growth at 5'C	-	Galactose	+
10^{o}C	-	Fructose	+
15^{o}C	+	Maltose	-
25^{o}C	+	Sucrose	-
30^{o}C	+	Rhamnose	-
37^{o}C	-	Arabinose	-
Growth in 0% NaCI	+	Amygdaline	-
Growth in 0.5% NaCI	+	Melibiose	-
Growth in 3% NaCI	+	Mannitol	-
Growth in 5% NaCI	-	Inositol	-
Cytochrome oxidase	+	Sorbitol	-
Catalase	+	Glycerol	-
Methyl red	+	Xylose	-
Voges-Proskaur	+	Lactose	-
Indole production	-	Trehalose	-
Nitrate production	-	Raffinose	-
Ammonium production	-	Cellobiose	-
Citrate production	-	Dextrin	-
H2S	-	Inulin	-
O/F	F	Glycogen	-
Gas from glucose	-	Adonitol	-
Arginine dihydrolase	+	Inositol	-
Lysine decarboxylase	-	Dulcitol	-
Ornithine decarboxylase	-	Eryhtritol	-
Tryptophan deaminase	-	Salicin	-
B-galactosidase (ONPG)	-	Aesculin	-
Urease	-		

Table 4.1. Characteristics of isolates of *Photobacterium damselae* subsp. *piscicida* (from Janssen and Surgalla, 1968;Yasunaga et al., 1983, 1984;Toranzoftal. 1991)

4.3.3 Serological Classification

Serological classification among *P. damselae* subsp. *piscicida* isolates from EU, USA and Japan revealed no differences based on cross-agglutination test (Toranzo et al, 1991). Similar results were obtained when monoclonal antibodies were applied (Bakopoulos et al, 1997), which is why other techniques mainly based on genetic analysis are employed to differentiate the isolates.

4.3.4 Molecular Classification

Due to high similarities in the biochemical and serological characteristics of *P. damselae* subsp. *piscicida*, molecular studies helped differentiate this bacterium based on plasmid size, random amplified polymorphism, sialic acid size (Jung et al, 2001a), and fragment length polymorphism of the 16S rRNA gene sequences (Kvitt et al, 2002). But the intergenic spacer region (ITS-2) of 16S and 5S rRNA, as well as the 16S and 23S rRNA intergenic spacer, was showed to be similar between *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* (Osorio et al, 2005).

4.3.5 Pathogenesis

The *P. damselae* subsp. *piscicida* route of infection is generally unknown. In the gills, it is assumed that the bacterium infect by attaching either to cell surface and/or mucus, an action that also triggers host nonspecific immune reactions. *P. damselae* subsp. *piscicida* has a variety of virulence factors which may help them evade host immune responses. Cell surface hydrophobicity combined with sugar binding capacity (Jung et al, 2001; Nagano et al, 2011b), capsular layer (Arijo et al, 1998), and LPS (Bonet et al, 1994) play important roles in initially adhering and invading cells followed by systemic host infection. The intracellular phase of *P. damselae* subsp. *piscicida* may be a mechanism to delay or avoid phagocytosis and host immune response, favoring the spread of infection and also help in the persistence and establishment of a carrier state in host (Acosta et al, 2009). Macrophages play a central role in immune response by phagocytizing and presenting the antigens to lymphocytes, but *P. damselae* subsp. *piscicida* induces apoptosis for macrophages and neutrophils simultaneously, rendering them incapable of killing the bacteria (do Vale et al, 2007; Noya and Lamas, 1997). This bacterium can also survive and replicate in the macrophage as an intracellular pathogen (Elkamel et al, 2003).

Extracellular products (ECPs) increase bacterial survival via a variety of ways, such as iron chelation and defending bacteria against host immune response. ECPs of *P. damselae* subsp. *piscicida* do not show strict host specificity but were strongly toxic for fishes and homoisothermic cell lines, has high phopholipase activity and displayed haemolytic activity for sheep, salmon and turbot erythrocytes (but not for trout erythrocytes) (Magariños et al, 1992). In sea bass, ECPs produced *in vivo* showed higher toxicity than the ECPs produced *in vitro*. Histologically, inflammatory and necrotic lesions in the spleen, liver, head, kidney, intestine, and heart can be observed post-introduction of the ECPs (Bakopoulos et al, 2004).

Nitric oxide (NO) is an effective bactericidal agent. However, capsulated strains showed

higher resistance to NO and peroxynitrites than the non-capsulated strains, suggesting that fish cannot produce NO that can effectively eliminate *P. damselae* subsp. *piscicida* (Acosta et al, 2004). Superoxide anion like NO, is also an effective bacteria-killing substance produced by macrophages but *P. damselae* subsp. *piscicida* produce superoxide dismutase (SOD) as a periplasmic catalase, which can resist the host superoxide anion (Díaz-Rosales et al, 2006).

Mobile genetic elements, a type of DNA that can move around within the genome and encode multiple antibiotics-resistance genes such as the *V. cholerae* SXT element, was also found in *P. damselae* subsp. *piscicida* (Osorio et al, 2004).

Iron is very important for the pathogenesis of *P. damselae* subsp. *piscicida* strains. Expression of capsular polysaccharide is dependent on iron availability and growth phase. Dietary iron increases susceptibility of sea bass to the pathogen (Rodrigues and Pereira, 2004). The outer membrane proteins of virulent *P. damselae* subsp. *piscicida* (EU) strains are capable of binding to haemin, and its binding activity is increased by iron limitation. The mechanisms rely mainly on the direct interaction between the haemin molecules and surface-exposed outer membrane protein receptors. Bacteria have developed a cascade of iron scavenging and transport systems, one such regulatory gene is Fur (ferric uptake regulator) repressor protein, which was cloned, identified and characterized in *P. damselae* subsp. *piscicida* (Osorio et al, 2004). In bacteria, siderophores, low molecular mass iron-chelating molecules, are used to remove iron from host iron-binding proteins. But *P. damselae* subsp. *piscicida* can acquire iron through a siderophore-independent mechanism via outer membrane receptor that transports the haem molecule into the periplasm through a TonB-dependent process. These haem uptake mechanisms are believed to contribute to virulence in fish (Lemos and Osorio, 2007). Furthermore, bacterial strains grown in iron depleted medium showed high antibody levels and different molecular weights of antigenic bands compared to those in glucose rich medium (Jung et al, 2007). On the other hand, bacteria isolated from the peritoneal cavity of sea bass had a different growth rate, smaller, produced a capsular layer, and possess different molecular weights of antigenic bands compared to those cultured *in vitro* (Jung et al, 2008) which might explain why *P. damselae* subsp. *piscicida* vaccine is difficult to develop.

4.4. Diagnosis

4.4.1 Clinical Signs

Clinical signs of pasteurellosis exist in both acute and chronic forms. In the acute form, high levels of mortality occur with no obvious symptoms of infection, including discoloration/black pigmentation and anorexia (Hawke et al, 2003), swelling in the abdominal cavity, and small hemorrhages around the gill covers or at the bases of the fins. No symptoms are evident during chronic infection except for constant low level mortalities.

4.4.2 Gross pathology

Gross pathological signs in pasteurellosis vary according to the form of the disease and

the species infected (Balebone et al, 1992). Signs in the acute form include pale liver and kidney, enlarged spleen and kidney, microerythemas with petechiae on organs and tissues, and occasional whitish nodules in the spleen. The latter characterize the chronic form of the disease and the reason why the infection is also called pseudotuberculosis.

4.4.3 Histopathology

Histopathological examination of white spotted lesions within visceral organs of diseased yellowtail suggests that these lesions are bacteria colonial lesions and granulomatous lesions. The early stages of infectious lesions are comprised mainly of many intracellular bacterial colonies. Enlarged lesions are comprised of many intracellular bacterial colonies, free bacterial cells and necrotic tissues. Small-sized bacterial colonies are often surrounded by accumulated macrophages forming nodular lesions. These nodular lesions develop into granulomas in which epithelioid cell layers encapsulate bacterial colonies and tissues showing coagulation necrosis. Within old granulomas, bacteria are markedly reduced. Diseased fish usually die of functional failure due to increased bacterial load and/or granulomatous lesions in visceral organs (Kubota et al, 1982).

On the other hand, diseased sea bass, sea bream and sole in Europe showed enlarged spleens with whitish lesions (Kakizaki et al, 1996). Histopathologically, the lesions are observed as focal necrosis with extracellular bacterial colonies in the spleen and haematopoietic tissues in the kidney. In the lesions, tissue necrosis is obvious and bacteria are propagated extracellularly with slight bacteria-phagocytosis by macrophages and neutrophils.

4.5. Diagnostic Methods

A common method to detect pasteurellosis is to check for gross pathological signs such as granulomatous-like white spots in the kidney and spleen.

In Japan, direct hybridization, using species-specific DNA fragment probe cloned from the chromosomal DNA was able to detect a number of pathogens from water and fish by colony hybridization. The plasmid identified from Japanese strain was used to diagnose and differentiate it from the EU isolates. Pulsed-field gel electrophoresis (Kijima-Tanaka et al, 2007), immunopolymerase chain reaction and oligonucleotide DNA array (Matsuyama et al, 2006) were also used for detection.

In EU, rapid detection of pasteurellosis can be done using latex agglutination test (BIONOR Mono-kit). The latex kit reacts with all strains of *P. damselae* subsp. *piscicida* with no cross-reactions with *V. anguillarum* and *P. multocida, P. haemolytica* and *Haemophilus parasuis* (Romalde et al, 1995). The magnetic bead-EIA (enzyme immunoassay) meanwhile, could also detect the strain but it showed cross reaction with *P. damselae* subsp. *damselae* and *P. histaminum* at high concentrations (10^9 to 10^{10} bacteria/ml). So far, the enzyme-linked immunosorbent assay (ELISA) is consideted as the most sensitive and rapid method (Bakopolous et al, 1997b).

Other diagnostic methods includes: PCR method using primers for16S rRNA gene sequence; by slide agglutination test using anti whole *P. damselae* subsp. *piscicida* cell

sera PCR; colony hybridization using *ureC* gene found only in *P. damselae* subsp. *piscicida* strains; random amplification of polymorphic DNA (RAPD) genomic fingerprints (Rajan et al, 2003); fluorescent antibody technique; detection of the capsular polysaccharide gene ; plating method using thiosulfate citrate bile salts-sucrose agar; and multiplex PCR and/or reverse line blot hybridization (Chang et al, 2009 ; López et al, 2012).

4.6. Prevention and Control

4.6.1 Prevention

Sound farming practices help to avoid conditions which stress the fish and predispose it to disease. The elimination of marine trash fish and removal of moribund and/or dead fish also reduces the incidence of pasteurellosis outbreaks and prevent pathogen infection from spreading. Ultraviolet or ozone treatment can be used remove *P. damselae* subsp. *piscicida* from seawater (Sugita et al, 1992). The use of immunostimulants like the hen egg lysozyme and β-glucan increased the resistance to pasteurellosis in yellowtail and gilthead sea bream, respectively. However, other immunostimulants such as schizophyllan and scleroglucan and M-glucan, chitin, Freund's complete adjuvant and *V. anguillarum* bacterin (Kawakami et al, 1998) did not increase resistance of yellowtail to pasteurellosis.

4.6.2 Chemotherapy

Many chemotherapeutic agents exhibit high antibacterial activity aganist *P. damselae* subsp. *Pisicicida* including amoxicillin, ampicillin (AMP), bicozamycin benzoic acid, florfenicol (FF), flumequine, novobiocin, oxolinic acid (OA), piromidic acid, fosfomycin, sulfisozole, thiamphenicol and oxytetracycline (Martínez-Manzanares et al, 2008). These chemotherapeutics have been widely used for treatment of pasteurellosis in marine fish farms. For example, in Japan, treatment of yellowtail infected with *P. damselae* subsp. *piscicida* is permitted provided that the antimicrobial agents, dosages and withdrawal times of chemotherapeutics allowed by the Japanese Fisheries Agency are followed (Table 4.2). The recommended period for oral administration of each drug to fish is 5-7 days.

However, *P. damselae* subsp. *Pisicicida* strains resistant to a number of chemotherapeutants such as chloramphenicol (CM), furazolidone (NF), kanamycin (KM), sulfonamide (SU) and tetracycline (TC), appeared suddenly in yellowtail farms in 1980 (Aoki and Kitao, 1985). Infection caused by multiple drug-resistant strains has continued until today. Drug resistance was caused by transferable R plasmids detected in antibiotic resistant strains that encode several drug resistant genes including AMP, CM, FF, KM, SU, TC and/or trimethoprim. In EU, some isolates showed resistant to erythromycin, KM, SM and SU and TC and OA.

Chemotherapeutic agents	Route of administration	Dosage	Withdrawal time
Amoxicillin	oral	40mg/kg	5days
Ampicillin	oral	20mg/kg	5days
Fosfomycin calcium	oral	40mg/kg	15days
Florfenicol	oral	10mg/kg	5days
Bicozamycin benzoic acid	oral	10mg/kg	27days
Novobiocin	oral	50mg/kg	15days
Oxolinic acid	oral	30mg/kg	16days
Sulfisoxasole	oral	100-200mg/kg	10days
Thiamphenocol	oral	50mg/kg	15days

Table 4.2. Chemotherapeutic agents approved for pseudotuberculosis of yellowtail in Japan

4.6.3 Vaccine

Fish are generally vaccinated by injection, oral, immersion or spray method using formalin-killed cells or live attenuated cells. Selecting the appropriate delivery method is important to increase vaccine efficacy. Vaccination can be done singly or by combining two delivery methods, or by double or triple trials of a single method (Bakopolous et al, 1997b). In the case of pasteurellosis, many scientists for the past 30 years have been trying to develop effective vaccines against this disease. Among the vaccines developed were: formalin killed bacterin, but this type showed different effects depending on cultivation time, temperature and NaCl concentration; live attenuated vaccine including AroA mutant (Thune et al, 2003); ribosomal antigen vaccine, a potassium thiocyanate extract (PTE) and/or an acetic acid-treated naked bacteria (NB); lipopolysaccharide-mixed, chloroform-killed cell (Kawakami et al., 1997); oil-based vaccine (Gravningen et al, 2008); toxoid-enriched whole-cell vaccine; divalent vaccines between *P. damselae* subsp. *piscicida* and Vibrio species, between *P. damselae* subsp. *piscicida* and V. alginolyticus and between *P. damselae* subsp. *piscicida* and V. harveyi bacterins; and subunit vaccine (Ho et al, 2011).

In Japan, commercial vaccines for pasteurellosis have not been made available since 1981 but recently, divalent vaccine with formalin-killed bacterin *P. damselae* subsp. *piscicida* and *Lactococcus garvieae* has been marketed.

4.7. Recent Topics

Outbreaks of pasteurellosis have caused considerable economic impacts particularly to species of yellowtail in Japan and sea bass and sea bream in the Mediterranean area where water temperature is more or less warm. It has been isolated from a wide range of environments such as estuarine and marine water, sediment, and healthy aquatic animals. Recently however, the increase in the number of fish species infected by this pathogen and the detection of the disease in cold and subtropical water areas is a cause for concern

(Pedersen et al, 2009; Wang et al, 2012).

It has been known that *P. damselae* subsp. *Piscicida* is an opportunistic pathogen in fish and mammals. But infections to human are very rare. Two cases *P. damselae* subsp. *piscicida* which were fatal by developing multiple organ failure within 20–36 h after the onset of initial symptoms (Takahashi et al, 2008).

With increasing antibiotic resistance, this pathogen has high potential as zoonotic agent thus effective measures should be set to lessen its threat to the aquaculture industry. First, is to develop effective vaccines and to lobby the use of these vaccines to reduce reliance to antibiotics. Secondly, if unavoidable, the use of antibiotics should be strictly regulated in order to restrict the possible transfer of antibiotic resistance genes between different ecological niches.

Glossary

ECPs : Extracellular products,

ELISA : Enzyme-linked immunosorbent assay,

PTE : Potassium thiocyanate extract,

NB : Naked bacteria

5. VIBRIOSIS

Yukinori Takahashi and Jun-ichi Hikima

5.1. Synopsis

Vibriosis is the significant disease of marine fishes or migratory species like salmonids, eels and ayu sweetfish (*Plecoglossus altivelis altivelis*) in fresh water. The disease was firstly described in eel, which was called “Red pest” or “Red disease”. The typical symptoms are hemorrhagic ulcer on the skin and necrotic lesions in muscle and fins. The causative agents are *Vibrio anguillarum* and the other Vibrionaceae. The organisms consist of Gran-negative straight or slightly curved rods 0.5-0.8μm ×1.0-2.0μm. They are non spore-forming and motile by monotrichous or multitrichous. The optimum temperature, sodium chloride levels and pH on growth generally are 25-30^{o}C, 1.0-2.0% and 7-8 respectively. The organisms gave positive oxidase and catalase reaction, utilized glucose fermentatively and did not produce gas from carbohydrate. They are sensitive to vibriostatic agent 0/129 (a pteridine compound). The mol% G+C of the DNA is 42-48%. Here, vibriosis and its causative agent, pathogenesis, detection methods, and prevention and control will be discussed.

5.2. Introduction

Vibrio anguillarum was first reported to be pathogenic to fish in 1909 when Bergman described an outbreak of disease in eels from the Baltic Sea (Bergman, 1909). The disease was characterized by the appearance of bloody lesions in the musculature of the infected fish. Since Bergman’s original description, numerous outbreaks of disease due to vibrios

have been recognized among a variety of fish in the world (Hoshina, 1957; Muroga, 2004). *V. anguillarum*, *V. ordalii* and *V. salmonicida* are designated from closely related strains. *V. ordalii* appear to be the seriously pathogenic against salmonids, ayu sweetfish (*Plecoglossus altivelis altivelis*) and jacopever (*Sebastes schlgelii*) (Muroga, 2004; Schiewe et al, 1981). *V. salmonicida* was isolated from salmonid and the other fish such a cod in cold water (Hjeltnes and Roberts, 1993; Schiewe et al, 1981). The organisms of all serotypes are more pathogenic in salmonids than in the other fishes. *V. vulnificus* was the first to isolated from Japanese eel (Muroga, 2004; Egidius et al, 1986). The bacteria were also reported to be pathogenic to European eel and human.

V. alginolyticus is an opportunistic pathogen or serves as a secondary invader of traumatized fish. *V. parahaemolyticus*, known to be a bacterium involved in food poisoning, was isolated from cultured yellowtail (*Seriola quinqueradiata*) (Muroga, 2004; Colorni et al, 1981). Vibriosis caused by *V. penaeicida* in cultured shrimp has been observed since 1980, and was known to have caused considerable loses to shrimp farms in Japan and New Caledonia (Hatai et al, 1975; Takahashi et al, 1985 ; Ishimaru et al, 1995 ; Costa et al, 1998).

5.3. Disease Agent

5.3.1. Characteristics

Table 5.1 shows the biological and biochemical characteristics of *Vibrio* species pathogenic to fish. The species consists of Gram-negative straight or straightly curved rods (Figure 5.1). They are non spore-forming and motile by monotrichous or multitrichous. Metabolism is aerobic or facultatively anaerobic and carbohydrates are fermented with the production of acid but not gas.

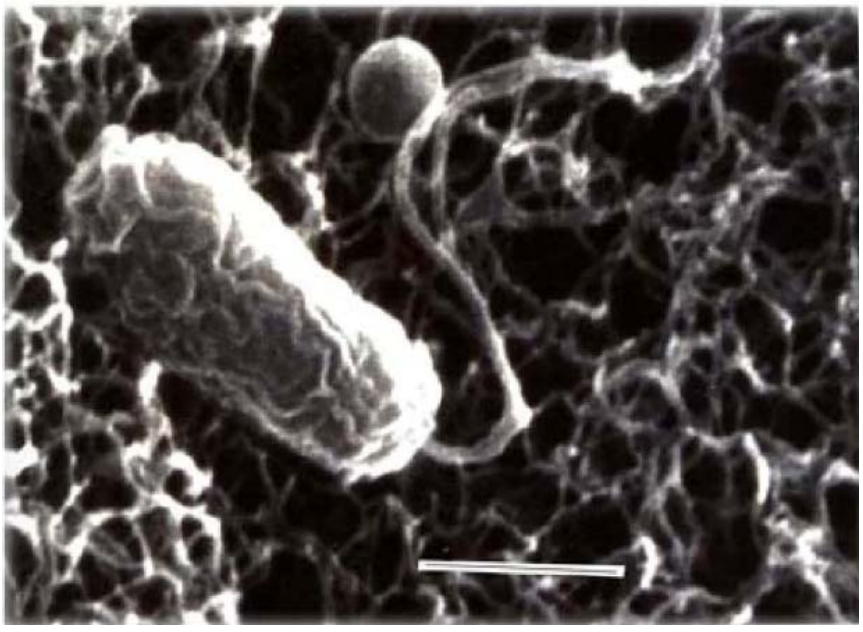

Figure 5.1. Electron microscopic photograph of *Vibrio anguillarum* (scale bar; 1.0 μm).

V. anguillarum typically grows within 24h at 25 ^{o}C on nutrient agar or TSA as a circular, raised, yellow-brown, opaque colony of 2 to 4mm. Growth on the media was observed at 10 to 35^{o}C with NaCl concentrations of 0.5 to 6.0 or 7.0%. No growth was observed in the media with 8.0% NaCL concentration.

This bacterium gives positive Voges-Proskauer reaction, gelatin liquefaction, nitrate reduction arginine hydrolysis, utilization of sucrose, and does not produce lysine, ornithine decarboxylase. The mol% G+C of the DNA is 44-46 (Muroga, 2004).

Characteristics	***Vibrio anguillarum***	***V. parahae-molyticus***	***V. alginolyticus***	***V. ordalii***	***V. salmonicida***	***V. vulnificus***	***V. penaeicida***
Swarming	-	-	+	-	-	-	-
Grows at 35ºC	+	+	+	-	-	+	-
Grows at 40 ºC	-	+	+	-	-	-	-
NaCl tolerance: 6%	+	+	+	+	-	-	-
NaCl tolerance: 8%	-	+	+	-	-	-	-
Gas from glucose	-	-	-	-	-	-	-
VP reaction	+	-	+	-	-	-	-
Gelatinase	+	+	+	+	-	+	+
Nitrate reduction	+	+	+	+	-	+	+
Arginine hydrolysis	+	-	-	-	-	-	-
Lysine decarboxilization	-	+	+	-	ND*	+	-
Ornithine decarboxilization	-	+	+	-	ND*	+	-
Utilize sucrose	+	-	+	+	-	-	-
DNA G+C mol%	44~46	46~47	46~47	43~44	42	46~48	46.2~47.0

Table 5.1. Biological and biochemical characteristics of fish pathogenic *Vibrio* species.

V. ordalii grows after 4 to 6 days at 22 on TSA as a white, circular, convex, translucent colony of about 1-2mm. Growth on the media is observed at 15-30^{o}C and NaCl concentrations of 0.5-5.0 or 6.0% (Schiewe et al, 1981). This bacterium gives positive gelatin liquefaction, nitrate reduction, utilization of sucrose and did not produce arginine hydrolase, lysine, orninthine decarboxylase (Schiewe et al, 1981). The mol% G+C of the DNA is 43-44 (Schiewe et al, 1981).

V. salmonicida has low growth temperature, 1-22^{o}C with an optimum of 15^{o}C. Some distinguishing features of the organisms are negative Voges-Proskauer reaction, gelatin liquefaction, nitrate reduction, arginine hydrolysis, lysine, ornithine decarboxilization, utilization of sucrose (Schiewe et al, 1981). The mol% G+C of the DNA is 42.

V. vulnificus grows rapidly within 24h at 25°C on nutrient agar as a circular, raised colony of 3 to 5mm. It is observed to grow in temperatures from 18 to 39°C and media with NaCl concentrations of 0.1 to 4.0%. This bacteria displays negative Voges-Proskauer reaction, arginine hydrolysis and utilization of sucrose (Egidius et al, 1986; Nishibuchi et al, 1979).The mol% G+C of the DNA is 46-48.

V. alginolyticus swarms grow on nutrient agar, TSA and BHI media. Growth of *V. alginolyticus* and *V. parahaemolyticus* are observed at 10 to 40°C. Growth occurred in media with NaCl concentrations of 0.5 to 7.0 or 8.0%. *V. alginolyticus* gives positive Voges-Proskauer reaction, gelatin liquefaction, nitrate reduction, lysine, ornithin decarboxilization, utilization of sucrose and do not produce arginine hydrolase (Hjeltnes and Roberts, 1993; Muroga, 2004). The mol% G+C of the DNA is 46-47. *V. parahaemolyticus* displays negative Voges-Proskauer reaction, arginine hydrolysis and utilization of sucrose (Muroga, 2004).

Shrimp pathogenic *V. penaeicida* grows within 24 to 48h at 25°C on ZoBell's agar as a circular, raised, whitish opaque colony of 0.5 to 1.0mm. Growth on the media is observed at 10 to 30°C with an optimum of 25°C and NaCl concentrations of 1.5 to 4 or 5%. The bacterium gives positive gelatin liquefaction, nitrate reduction and do not produce arginine hydrolase, lysine, ornithine decarboxylase (Hatai et al, 1975; Takahashi et al, 1985). The mol% G+C of the DNA is 46.2-47,0 (Takahashi et al, 1985).

5.3.2. Serological Classification

The protein and lipopolysaccharides in the membrane of *V. anguillarum* have been extensively characterized in the eight (J-0-1~J-0-8) or sixteen (01~016) different O serogroups identified among Japanese strains or the pathogenic strain from marine fish (Saulnier et al, 2000 ; Tajima et al, 1985).

The Serological types of *V. ordalii* are in accord with the serotype J-0-1 of *V.anguillarum* (Muroga, 2004). The organisms strains showed 58 to 69% relatedness to *V. anguillarum* by analysis of DNA-DNA hybridizations (Schiewe et al, 1981).

V. salmonicida have two distinct serotypes: salmonids or the other fish pathogenic strains. The organisms was distinguished from *V. anguillarum* and *V. ordalii* by DNA-DNA hybridization (Grisez and Ollevier, 1995).

5.3.3. Genome Sequence

To date, the complete gemone of only one species, *V. anguillarum* 775 strain (isolate from coho salmon *Onchorhynchus kisutch*; ATCC 68554), has been determined among *Vibrio* species.[22)] Meanwhile, draft sequences of *V. anguillarum* 96F strain (isolate from striped bass, *Morone saxatili*s), RV22 (isolate from turbot, *Scophthalmus maxinus*) and *V. ordalii* ATCC 335509 (isolate from coho salmon) are also published in GenBank genome database (GenBank Assembly IDs: GCA_000217675.1, GCA_000257165.1, GCA_000257185.1, GCF_000257205.1).

The complete genome sequence of *V. anguillarum* 775 consists of two chromosomes,

3,063,912 bp chromosome 1 (Chr1) and 988,135 bp chromosome 2 (Chr2), in addition to the 65,009 bp pJM1 plasmid. Chr1 and Chr2 contain 2,864 and 951 annotated genes, respectively. The majority of genes for essential cell functions and pathogenicity are located on Chr1. In constract, Chr2 contains a larger fraction (59%) of hypothetical genes than does Chr1 (42%), and also harbors a superintegron, as well as host addiction genes that are typically found in plasmids (Wiik and Egidius, 1986).

5.3.4. Pathogenesis

The extracellular protease of *V. anguillarum* was demonstrated as a factor of the severe focal myonecrosis and liquefaction of fish musculature (Naka et al, 2011; Stensvåg et al, 1993). The pathogenesis of the fish pathogenic *Vibrio* to produce hemolytic anemia, which results in high circulating and melano-macrophage related iron levels in affected fish, is related to its high requirement for iron. The pathogenic strains have a well-developed iron sequestering mechanism based on secretion of a siderophore, which induces separation of plasma and tissue iron from its transferrin or ferritin binding proteins. They form complexes to the siderophore and attaches to specific transport outer cell membrane proteins for absorption into the bacterial cells (Morita et al, 1996; Roberts, 1975).

5.3.5 Virulence Factors and the Coding Genes

V. anguillarum virulence is associated with the presence of a plasmid-mediated iron uptake system, which is involved in an efficient iron-sequestering system consisting of the indigenous siderophore anguibactin (Actis et al, 1985; López and Crosa, 2007). In *V. anguillarum, angB/G*, *angM*, *angN*, *angR* and *angT* genes, which code for non-ribosomal peptide synthetasea (NRPSs), catalyse the synthesis of anguibactin (Actis et al, 2011). In addition, the genes *fatA*, *fatB*, *fatC* and *fatD* are involved in the transport of ferric-anguibactin complexes. These transport genes, together with the biosynthesis genes *angR* and *angT*, are included in the iron-transport biosynthesis (ITB) operon (López and Crosa, 2007).

Other virulence factors in *Vibrio* species such as heamolysin, flagellum and heamin-uptaking factors have been known. Haemolysin is a protein that causes lysis of red blood cells by damaging their cell membranes. In *V. anguillarum*, there are four heamolysin genes: vah2, vah3, vah4 and vah5. These ORFs encode 291, 690, 200 and 585 amino acid residues, and were found to be 33, 75, 22 and 66 kDa in size, respectively. Homologues of these genes were also found in *V. vulnificus* and *V. cholerea* (López and Crosa, 2007).

Flagellum is also involved in the virulence of *V. anguillarum* (Di Lorenzo et al, 2003). The product of flagellin gene (*flaA*) is needed for crossing the fish integument and might play a role in virulence (Chart, 1983). The genes, *virA* and *virB* encoding a major surface antigen are important for the virulence of *V. anguillarum* (Milton et al, 1996). This surface antigen appears to be a lipopolysaccharide located on the outer sheath of the flagellum and is expressed *in vivo* during fish infections with the flagellum. Another gene, *virC* is also essential for virulence although the function of the product is still unknown (Norqvist and Wolf-Watz, 1993).

V. anguillarum can utilize heamin and heamoglobin as iron sources. Nine genes, *huvA*, *huvZ*, *huvX*, *tonB1*, *exbB1*, *exbD1*, *huvB*, *huvC* and *huvD*, encoding the proteins involved in heamin transport and utilization, are clustered in a 10-kb region of chromosomal DNA (Milton et al, 1995; Mouriño et al, 2004).

5.4. Diagnostic Methods

5.4.1 Clinical and Histopathological Signs

In juvenile fish, first signs of the vibriosis are often anorexia, abrasion on the skin or fins, darkening and sudden death. In young and adult stages, first signs of losses are erosious leukoplakia, haemorrhage on the skin and fins (Figure 5.2). The developmental symptoms are haemorrhagic ulcers on the mouth, head, trunk skin or else local necrotic lesions in the muscle (Figure 5.3) (Hjeltnes and Roberts, 1993; Muroga, 2004; Mouriño et al, 2006; Muroga, 1992). Other symptoms like exophthalmus, haemorrhage of periorbitis, reddening and expansion of anus are observed.

Figure 5.2. Ayu sweetfish infected with vibriosis caused by *Vibrio anguillarum*, showing the haemorrhage on the skin.

Figure 5.3. Yellowtail infected with vibriosis caused by *Vibrio anguillarum*, showing skin ulcer.

Internally, the main signs such as intestinal haemorrhage and congestion of the liver, spleen and kidney are seen in affected fish especially at progressed stages (Hjeltnes and Roberts, 1993; Muroga, 2004).

Histopathological signs are characterized by the skin lesions which comprise hypodermal inflammatory foci extending deep into the muscle. There is severe myofibrillar necrosis with the centre of the lesion comprising an agglomerate of sacroplasmic debris. There are local necrosis in the liver, spleen and kidney a depletion and necrosis of haemopoietic elements (Hjeltnes and Roberts, 1993; Muroga, 2004).

The typical symptoms of vibriosis by *V. penaeicida* in diseased shrimp are cloudines of the muscle at the 6th abdominal segment and brown spots in the gills and lymphoid organ (Figure 5.4) (Hatai et al, 1975). Histopathological characteristics include extensive necrosis caused by severe bacterial invasion and multiple formation of melanized nodules in the lymphoid organ (Kusuda and Salati, 1993).

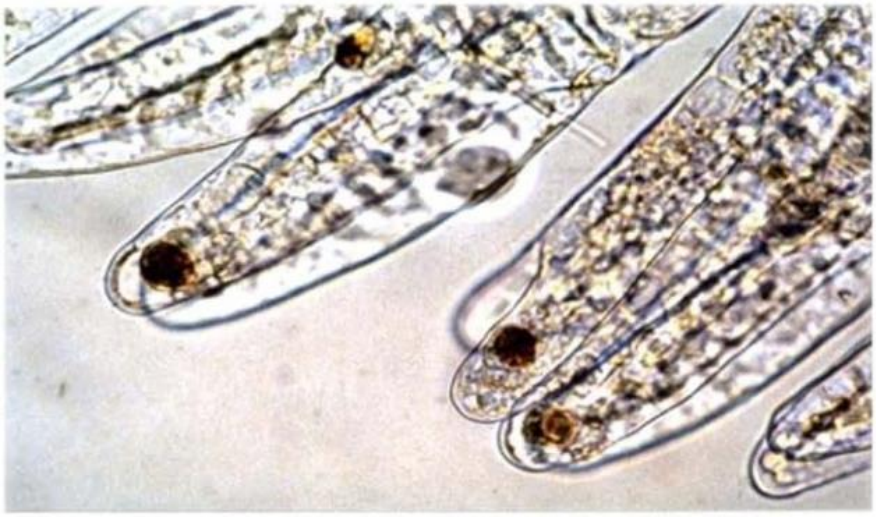

Figure 5.4. Shrimp infected with vibriosis caused by *Vibrio penaeicida*, showing brown spots in the gills.

5.4.2 Diagnosis

(2-1) Slide agglutination test
The rapid diagnostic test is usually performed to diagnose suspected culture of bacteria isolated in artificial culture. ①□One colony of a fresh culture of the organism is mixed with saline on a degreased glass slide in two separate drops. ② A drop of prepared anti-many serogroups of *Vibrio* species rabbit antiserum is added to one drop and a drop of saline to the other.

(2-2) Fluorescent antibody method
The method has advantages for the experienced researcher in rapid diagnosis of vibriosis. ① Smears or crystat sections of suspected tissue are fixed in acetone for 5min, the washed in phosphate buffered saline (PBS). ② A drop of prepared anti–many serogroups of *Vibrio* species rabbit antiserum or the immunoglobulin G (IgG) is added

on the tissue and incubated at room temperature for 30 min. ③ The antiserum (IgG) is washed off carefully with PBS and the slide washed three times with PBS. After air, drying a drop of fluorescein isothiocyanate labelled goat antiserum against rabbit globulin is added on the tissue, incubated for 30 min then washed with PBS. After several further washings the specimen is ready for examination.

(2-3) DNA hybridization method using a specific DNA probe

Diagnostic method	***Vibrio* species**	**Target gene**	**Reference**
Colony hybridization	*Vibrio anguillarum* (serotype A)	Chromosomal DNA (Randamly cloned DNA fragment, 562 bp)	32
	V. anguillarum, V. ordalii	5S rRNA gene (synthesized oligonucleotide)	44
Southern blot hybridization	*V. anguillarum*	16 and 23S rRNA gene (derived from *Escherichia coli;* merchandised by Boehringer Mannheim)	29
	V. anguillarum (serotype O1)	16 and 23S rRNA gene (derived from *Escherichia coli;* merchandised by Boehringer Mannheim)	30
	V. anguillarum	16 and 23S rRNA gene (derived from *Escherichia coli;* merchandised by Boehringer Mannheim)	31
PCR	*V. anguillarum*	Hemolysin gene (PCR products: 490 bp)	33
	V. anguillarum	*rpoS* gene (PCR products: 689 bp)	34
	V. anguillarum	*empA* gene (PCR products: 439 bp)	35
	V. anguillarum	*toxR* gene (PCR products: 93 bp)	36
	V. anguillarum	16S rDNA (PCR products: 81 bp)	36
	V. trachuri	Chromosomal DNA (Randamly cloned spcific region, 417 bp)	45
	V. vulunificu	Cytotoxin-hemolysin gene (PCR products: 519 bp)	37
	V. vulunificu	23S rRNA gene (PCR products: 978 bp)	39
	V. vulunificu	Cytolysin-hemolysin gene (PCR products: 1416 bp)	38
LAMP	*V. anguillarum*	metalloproteinase (*empA*) gene	41
	V. alginolyticus	gyrase B (*gyrB*) gene	42
	V. nigripulchritudo	Intergenic spacer region (ITS)	43

Table 5.2. Diagnostic methods for detecting *Vibrio* species in infected fish.

The basic principle of the DNA hybridization is the binding/hybridization of a single stranded DNA with its complementary DNA thereby forming a double stranded DNA. It is can be used to distinguish close species because the probe DNA labeled with a radioactive isotope or a fluorescent substance specifically hybridized with the nucleotide sequence encoding a target gene. In the DNA hybridization, there are three kinds of methods: colony hybridization, dot-blot hybridization and Sothern blot hybridization. The bacterial DNA fixed on a cellulose-nitrate or nylon membranes, and the labeled DNA probe is made to react in a hybridization solution, and then the radioactive isotope or fluorescent substance labeling the probe are detected by exposure on an X-ray film after washing.

Vibrio species detected using the DNA hybridization method have been reported (Table 5.2). *V. anguillarum* was detected by Southern bolt hybridization using 16S and 23S rRNA gene probe derived from *Escherichia coli*, which was commercialized by Boehringer Mannheim (Egusa et al, 1988; Pedersen et al, 1994). *V. anguillarum* serotype A was distinguished by colony hybridization using DNA probe encoding the serotype-specific gene (Skov et al, 1995).

(2-4) Polymerase chain reaction (PCR) method

PCR is a technology developed using the Taq DNA polymerase capable of working effectively in high temperatures (a heat-stable DNA polymerase) and allows for the amplification of a target gene. This method is described in detail in *Diagnosis-PCR Detection*. Because of its simplicity, quickness, and relatively higher accuracy compared with probe diagnostic methods, the PCR method is widely used for detection of specific genes of pathogenic bacteria. This method is also currently used to detect the causative bacteria for many fish and shellfish species.

PCR detection methods for Vibriosis have already been developed and used to successfully diagnose the presence of the causative agents of the disease. *V. anguillarum*, *V. trachuri*, and *V. vulunificu* have been detected by the PCR method (Table 5.2). Accurate and reliable diagnoses are made possible by amplifying genes that are specific to the bacteria such as hemolysin gene (Aoki et al, 1989), *rpoS* (Hirono et al, 1996), *empA* (Kim et al, 2008), *toxR* (Xiao et al, 2009), Cytotoxin-hemolysin gene (Crisafi et al, 2011; Hill et al, 1991) and 16S and 23S rRNA (Xiao et al, 2009; Coleman et al, 1996) (Table 5.2).

(2-5) Loop-mediated Isothermal Amplification (LAMP) method

The loop-mediated isothermal amplification (LAMP) reaction is an autocycling strand displacement DNA synthesis performed using a DNA polymerase with a high level of strand displacement activity and a set of specially designed inner and outer primers (Arias et al, 1995). The details of the principle are shown in "*Diagnosis: LAMP methods*".

The LAMP method has the following advantages when compared to the conventional PCR method: (1) since isothermal amplification of DNA is possible, this method does not need a thermal cycler; (2) the use of four different primers enhances its specificity; (3) the amplification efficiency of DNA is high (it amplifies 10^9 to 10^{10} times from 15 minutes in 1 hour); (4) the result can be visible without using electrophoresis (the LAMP products can be observed by adding pyrophoric acid magnesium into the reaction causing positive

samples to turn cloudy).

Target in genes in various Vibrio species including, *empA* in *V. anguillarum* (Notomi et al, 2000), *gyrB* in *V. alginolyticus* (Hongwei et al, 2010), and ITS (intergenic spacer region between 16S and 23S rRNA genes) in *V. nigripulchritudo* (Cai et al, 2010) were detected by LAMP method (Table 5.2).

5.5. Control

The prevention of vibriosis is best achieved by maintenance of environment, good husbandry and low stocking densities. However, in most cases such as clinical outbreaks, antibacterial agents are used for therapy. Oxytetracycline (OTC), sulphonamides (SA) or oxolinic acid (OA) are the most commonly used drugs. Oral-administration of OTC, SA or OA are used, with prescribed dosage of 50, 100-200, 20-30mg/kg fish 1day, respectively in for 5 days.

All of the licensed vaccines available are highly effective against salmonids vibriosis, which includes both *V. anguillarum* and *V. ordalii* infections (Hjeltnes and Roberts, 1993). In Norway, *V. anguillarum* and *V. salmonicida* combined vaccine is also available against salmonids (Hjeltnes and Roberts, 1993).

It has been reported that the inactivatied *V. anguillarum* vaccine is effective against infected ayu and salmonid fish by immersion method (Ellis, 1988; Mughal et al, 1986).In Japan, the immersion vaccination against salmonids, ayu sweetfish and yellowtail vibriosis are authorized by the government. As an antigen of the *Vibrio* vaccine, freezed-killed bacteria desolved in distilled water is effective by immersing as well as that of formalin-killed bacteria (Kawano et al, 1984). Furthermore, since efficacy of immersion vaccine using LPS extracted from *V. anguillarum* is also revealed in ayu, LPS is involved in antigen of *Vibrio* immersion vaccine (Aoki et al, 1984).

Glossary

Chr: Chromosome,

ITB: Iron-transport biosynthesis,

PBS: Phosphate buffered saline,

Ig: Immunoglobulin,

PCR: Polymerase chain reaction,

LAMP: Loop-mediated Isothermal Amplification,

ITS: Intergenic spacer region,

OTC: Oxytetracycline,

SA: Sulphonamides,

OA: Oxolinic acid

6. TENACIBACULOSIS

Hisatsugu Wakabayashi

6.1. Synopsis

The genus *Tenacibaculum*, within family Flavobacteriaceae, consist of Gram negative, long thin bacteria which are not flagellated and are motile by gliding when in contact with a solid surface. They grow well on low nutrient media containing sea water producing pale yellow rhizoid colonies. *Tenacibaculum maritimum* is one of the most threatening bacterial pathogens for a wide range of cultured marine fish species in the world. *Tenacibaculum discolor* and *Tenacibaculum soleae* are the isolates from diseased cultured sole (*Solea senegalensis)* in Spain. The diseased fish are similar in appearance to those with columnaris disease in freshwater fish. *Tenacibaculum ovoliticum* is an opportunistic pathogen for Atlantic halibut (*Hippoglossus hippoglossus*) eggs and larvae in Norway.

6.2. Introduction

Outbreaks of columnaris-like gliding bacterial diseases were reported in salmonid fishes cultured in sea water in the North America, but the causative agent was not able to be specified (Borg, 1960; Wood, 1974; Anderson and Conroy, 1969; Sawyer,1976). Masumura and Wakabayashi (1977) described outbreaks of a gliding bacterial disease in hatchery born red sea bream (*Pagrus major)* and black sea bream (*Acanthopagrus shlegeli)* fry reared in marine net-cages in Japan. The diseased fish were similar in appearance to those with columnaris disease and large numbers of gliding bacteria were observed in scrapings from lesions. An organism was isolated on Cytophaga agar (Anacker and Ordal 1959) prepared with seawater, and the disease condition was produced by experimental infections. The causative bacteria obligatorily required sea water for growth and this could not be replaced by NaCl alone (Hikida *et al*. 1979). Wakabayashi *et al*. (1986) proposed the name *Flexibacter maritimus* sp nov. for the organism. Subsequently *F. maritimus* infection was reported in Japanese flounder (*Paralichthys olivaceus*) reared in marine hatcheries in Japan (Baxa et al. 1986, 1987).

An investigation carried out by Bernardet and Grimont (1989) revealed that a strain of a '*Flexibacter columnaris*-like' bacterium depositied in the National Collection of Marine Bacteria (strain NCMB 2158) was synonymous with *F. maritimus*. This strain was originally reported as the etiological agent of 'black patch necrosis' (BPN) in Dover sole (*Solea solea*) by Campbell and Buswell (1982). Then, *F. maritimus* infection was reported by various marine fishes cultured in France and Spain (Alsina and Blanch, 1993; Pazos et al, 1993; Bernardet et al., 1994).

Chen and Henry-Ford (1995) isolated *F. maritimus* from the gill and skin lesions of chinook salmon (*Oncorhynchus tschawytscha*) and white sea bass (*Atractoscion nobilis*) reared in marine net-pens along the southern California coast. They also recovered *F. maritimus* in lesions of northern anchovy (*Engraulis mordax*) and Pacific sardine (*Sardinops sagax*) which were used as live bait. Handlinger et al. (1997) reported *F. maritimus* infection of cultured marine fishes, such as striped trumpeter (*Latris lineata*), salmonid fishes and greenback flounder (*Rhombosolea tapiria*) in Tasmania, Australia.

Kent et al. (1988) reported pathological changes and mortality associated with a *Cytophaga* infection in Atlantic salmon (*Salmo salar*) smolts maintained in net pens along the coast of Washington State, USA. Gliding bacteria were isolated on the seawater Cytophaga agar (SCA) from lesions, and were identified as a *Cytohpaga* sp. The organism was a marine bacterium requiring at least 10% seawater for growth, but serologically distinct from *F. maritimus*. Two new species of the genus *Tenacibaculum,* i.e. *Tenacibaculum discolar* and *Tenacibaculum soleae* were isolated from diseased cultured sole (*Solea senegalensis*) which showed the typical signs observed in fish affected by *T. maritimum* (Pineiro-Vidal *et al*., 2008a; Pineiro-Vidal *et al*., 2008b). A psychrotrophic *T. ovolyticum* (syn. *Flexibacter ovolyticus*) was isolated for the adherent bacterial epiflora of Atlantic halibut (*Hippoglossus hippoglossus)* eggs and was shown to be an opportunistic pathogen for halibut eggs and larvae (Hansen et al., 1992).

6.3. Disease Agent

The gliding bacteria isolated from diseased sea breams in Japan was thought as a new species of *Flexibacter*, for which the name of *Flexibacter maninus* was mooted (Hikida et al., 1979). Subsequently, Wakabayashi et al., 1986) formally proposed the name of *Flexibacter maritimus* to accommodate the pathogens. Although renaming the organism *Cyophaga marina* was proposed by Reichenbach (1989), it was judged that *F. maritimus* was a priority species name (Holmes, 1992). Suzuki et al. (2001) pointed out that *F. maritimus* and *F. ovolyticus* were genetically misclassified bacteria, because these species were distantly related to *Flexibacter flexilis*, the type species of the genus *Flexibacter*. They proposed the reclassification of *F. maritimus* and *F. ovolyticus* into *Tenacibaculum* gen. nov., as *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov.

T. maritimum has an absolute requirement for sea water. No growth occurs on cytophaga agar (Anacker and Ordal, 1959) with the addition of NaCl instead of seawater. At least 30% seawater is required. The bacterium requires KCl as well as NaCl for growth. Ca^{++} enhances growth while SO^{++} is slightly inhibitory (Hikida et al., 1979). In cultivation of *T. maritimum*, seawater cytophaga agar (Masumura and Wakabayashi, and 1977), TYC (Hikida et al., 1979), Marine 2216E (Difco) (Bernardet et a. 1994), FMM (Pazos et al., 1996) and SFM (Bullock et al., 1986) have been used. Colonies on seawater cytophaga agar are pale yellow, flat and thin with uneven edges and adherent to the agar (Figure 6.1). The pigment is not flexirubin-type. In un-agitated liquid medium, surface growth is in the form of a pellicle. The growth occurs from 14.6 to 34.3^{o}C. Bacterial cells from fresh culture are Gram-negative, flexible slender rods (0.3-0.5 x 2-30 μm). As the cultures age, however, the cells tend to become somewhat shorter and produce round bodies. These spherical cells are not capable of germinating in fresh medium. The organisms have no flagella but exhibit gliding motility on a wet surface. Although columnar formation is not so obvious as in *F. columnare*, *T. maritimum* also gathers into masses on the periphery of isolated tissues on wet mount preparations (Figure 6.2). *T. maritimum* produces catalase, cytochrome oxidase and ammonium and hydrolyses casein, gelatin, tributyrin and tyrosin. It does not produce hydrogen sulphate or indole. Nitrogenous compounds such as tryptone, yeast extract and casamino acid are utilized as sources of carbon and nitrogen for growth. Agar, cellulose, chitin, starch and aesculin are not degraded. Nitrate is reduced to nitrite. Acid is not produced from glucose, galactose, fructose, mannose,

lactose, sucrose, sorbose, maltose, cellobiose, trehalose, xylose, rhamnose, raffinose, dextrin, glycogen, inulin, glycerol, adonitol, sorbitol, inositol or salicin. The G+C ratio of the DNA is a 31.3-32.5mol % (Hikida et al. 1979; Wakabayashi et al. 1986).

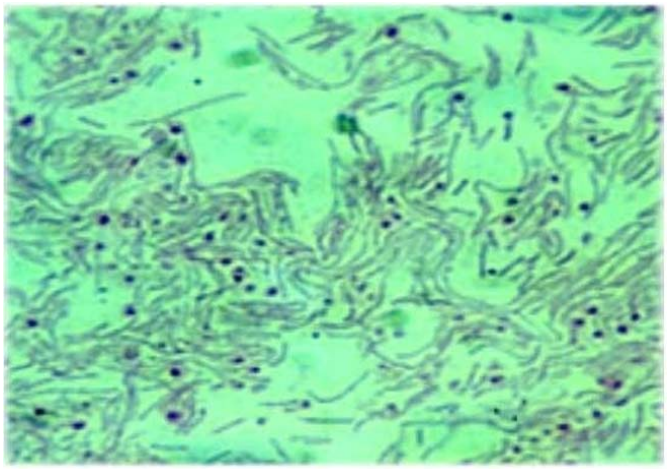

Figure 6.1. *Tenacibaculum maritimum* cells pure-cultured on Seawater Cytophaga broth, showing a mixture of slender rods and round bodies.

T. ovolyticum is Gram-negative, long, slender rods (0.4 x 2-20 μm) which occasionally grow to filaments that are 70 to 100μm long. Colonies are Kovacs oxidase positive and pale yellow. Microcysts are not formed. The cells exhibit gliding motility, do not adsorb Congo red, and do not possess a flexirubin type of pigment. It is strictly aerobic and does not produce acid from carbohydrates. Gelatin, tyrosine, DNA, and Tween 80 are degraded, but starch, cellulose, and chitin are not degraded. It possesses catalase and nitrate reductase activities. H_2S is not produced, and 50% seawater is required for growth. *T. ovolyticum* grows at 4°C, but not at 30°C. The G＋C ratio of the DNA is a 30.3-32.0 mol% (Hansen et al., 1992)

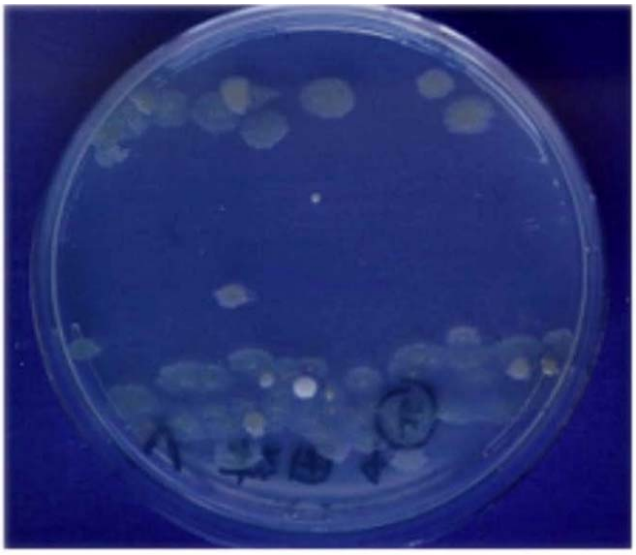

Figure 6.2. Colonies of *Tenacibaculum maritimum* isolated from a lesion tissue on Seawater Cytophaga agar, including a few other bacterial colonies.

Agglutination tests using polyclonal antisera showed cross-reaction for all strains regardless of host species and serum employed, and the heterologous titers were similar to homologous ones (Wakabayashi et al., 1984; Ostland et al., 1999; Avendano et al., 2004). Immunodifusion and western immunoblot analysis demonstrated distinct antigenic differences among the strains (Ostland et al., 1999).

In accordance with the results obtained with absorbed antisera, the strains were provisionally divided into 2 serological groups (Avendano et al., 2004). Immunoblot analysis of the LPS clearly revealed serotypes O1 and O2 that were distinguishable without the use of absorbed antiserum (Avendano et al., 2004) Avendano et al. (2005) reported the existence of a new serotype, which was proposed as serotype O3. These serotypes mainly associated with the host species (Avendano et al., 2005)

The ability of *T. maritimum* to produce disease in various fish species was examined by many authors and mortalities among experimental fish varied widely depending on the method of infection. The disease was not induced by intramascular or intraperitoneal injection of the pathogens in experimentally infected red and black sea bream, sea bass or turbot (Wakabayashi et al., 1984; Alsina & Blanch, 1993; Pepin & Emery, 1993; Bernardet et al., 1994; Avendano et al., 2006). Fatal infections occurred most frequently when fish were exposed to topical application of the culture on the surface of the mouth or tail (Wakabayashi et al., 1984). However, Campbell & Buswell (1982) challenged two group of 10 Dover sole with a bacterial suspension. The group challenged by scarification showed no signs of the disease, but the group injected subdermally had a 30% mortality after 48h. Bath challenge was not a reliable method of inducing the disease in sea bass unless the skin is previously scarified (Wakabayashi et al., 1984; Baxa et al, 1987; Bernardet et al., 1994) or the bacterial strain is first passaged twice in Atlantic salmon (Handlinger et al., 1997). Avendano et al., (2006) demonstrated that, using prolonged immersion of turbot for 18h with the pathogen at 18 to20^{o}C, the disease could be easily reproduced with the fish showing the classical signs of the disease.

6.4. Diagnostic Methods

In the case of the fry of red sea bream or black sea bream, the clinical signs of *T. maritimum* infection are loss of appetite, lethargy and darkening of the body surface. The diseased fish swim abnormally near the edge of net cage. They have eroded mouths, frayed fines and tail rot (Figure 6.3). In the lesions large numbers of long, slender rod-shaped bacteria are observed and give the infected tissue a pale yellow appearance. In older fish the lesions occur initially as grey-white cutaneous foci on the fins, head and trunk. On the skin, the lesions become eroded and shallow ulcers are produced (Masumura and Wakabayashi 1977, Wakabayashi et al. 1984). McVicar and White (1979) described the clinical signs of 'black patch necrosis' in Dover sole as slight blistered of the skin surface or darkening of tissue between caudal and marginal fin rays followed by extensive darkening of the area, loss of the epithelial surface and hemorrhage in exposed dermal tissues. Similar clinical signs of *Tenatibaculum* infection were reported on many kinds of fishes, i.e. Japanese flounder (Baxa et al. 1986), Atlantic salmon (Kent et al. 1988; Handlinger et. al. 1997, Ostland et al. 1999, Olsen et al. 2011), turbot (Alicina and Blanch 1993), sea bass (Bernardet et al., 1994), white sea bass (Chen et al., 1995), Pacific sardine (Chen et al., 1995), Chnook salmon (Chen et al., 1995),

rainbow trout (Handlinger et al. 1997), green flounder (Handlinger et al., 1997), striped trumpeter (Handlinger et al. 1997), wedge sole (Lopez et al., 2009), Senegal sole (Vilar et al. 2012), and Asian sea bass (Gibson-Kueh et al., 2012). The presumptive diagnosis is based on the clinical signs of the diseased fish, particularly gross external lesions, as well as in the microscopic examination of the lesions revealing long, thin, rod-shaped bacteria which show gliding motility in wet mounts.

Figure 6.3. Tail rot caused by *Tenacibaculum maritimum* in juvenile black sea bream (upper) and red seabream (lower). Eroded mouth and frayed fins are also observed.

Figure 6.4. Cells of *Tenacibaculum maritimum* swarming on the margin of eroded fin tissues.

Diagnosis can be confirmed by the isolation of bacterial colonies on an appropriate medium such as seawater cytophaga agar, TYC, Marine 2216E, FMM, or SFM, followed by morphological and biochemical characterization. This traditional method is time consuming and somewhat difficult to distinguish *T. maritimum* from other phenotypically similar species. In addition, due to the slow growth characteristic of *T. maritimum*, the mixed other bacterial species sometimes overgrow the *T. maritimum* colonies. The development of polymerase chain reaction (PCR)-based methods offer the possibility of

more rapid and accurate identification of the pathogen from plate cultures as well as from tissues. Toyama et al. (1996) designed a pair of primers MAR1 (5'-AATGGCATCGTTTTAAA-3') and MAR2 (5'-CGCTCTCTGTTGCCAGA-3') for the detection of *T. maritimum* using 16S rRNA gene as target. Bader and Shotts (1998) also selected a pair of *T. maritimum* species-specific PCR primers Mar1 (5'-TGTAGCTTGCTACAGATGA-3') and Mar2 (5'-AAATACCTACTCGTAGGTACG-3') targeted 16S rRNA gene. In order to increase the sensitivity, two nested PCR protocols for detection of *T. maritimum* based on MAR1-MAR2 and Mar1-Mar2 were developed (Avendano-Herrera et al., 2004a,c; Cepeda et al. 2003). PCR-enzyme-linked immnosorbent assay (PCR-ELISA)(Wilson et al., 2002), reverse transcriptase polymerase chain reaction-enzyme hybridization assay (RT-PCR-EHA)(Wilson and Carson 2003) and DNA microarray probe (Warsen et al. 2004) are also available for the direct detection of *T. maritimum* from pure culture.

6.5. Control

T. malitimum is sensitive to various antibiotics *in vitro* studies (Alicna and Blanch 1993, Pazos et al. 1993, Chen et al., 1995, Soltani et al. 1995. Avendano-Herrera et al. 2005) and therefore any approved drug can be used. However, field results are not always satisfactory even if the isolated bacteria are highly sensitive to the drug (McVicar and White, 1979, Handlinger et al 1997, Sepeda and Santos 2002).

As with other infections, avoiding overcrowding and overfeeding is recommended, particularly during the expected time of outbreak (McVicar and White 1982, Wakabayashi 1993, Soltani et al 1996). McVicar and White (1979, 1981) demonstrated that the addition of a sand substrate to tanks would rapidly cure the disease in juvenile Dover sole and remarkably improve hatchery survivals.

Development of a vaccine has been pursued in Spain and Australia (Tranzo et al. 2005, van Gelderen et al. 2009), but no practical, commercially available vaccine has been marketed.

6.6. Recent Topics (Jellyfish as a vector of *T. maritimum*)

Ferguson et al. (2010) demonstrated that *T. maritimum* was present on the mouth of jellyfish, such as *Phialella quadrata* and that their DNA sequences were almost identical to those of *T. maritimum* present on the gill of farmed Atlantic salmon in Scotland. They thought that the initial damage to gills, likely produced by nematocyst-derived toxins from the jellyfish, was compounded by secondary bacterial infection with *T. maritimum*, and that *P. quadrata* was probably acting as a vector for the pathogen. By the use of real-time PCR, *T. maritimum* DNA was detected at low level in four of 26 jellyfish, three *P. quadrata* and one *Muggiaea atlantica*, which were collected in summer 2010 from seawater locations around Ireland (Fringuelli et al. 2012). Several interesting questions were raised (Ferguson et al. 2010). What is the relationship between the bacteria and the jellyfish? Are they a pathogen of the jellyfish, or are they present in a symbiotic or commensal capacity?

7. EDWARDSIELLOSIS (EDWARDSIELLA TARDA)

Tomokazu Takano

7.1. Synopsis

Edwardsiella tarda causes a serious systemic infection in fish that are characterized by septicaemia and necrotic abscesses. *Edwardsiella tarda* infects a variety of marine and freshwater fish species, and at least 40 species have been recorded as hosts of this bacterium (Evans et al, 2011). Amongst economically important marine aquaculture species, flatfish, bream and sea bass are seriously affected by *E. tarda* infections. On the other hand, there are few reports of *E. ictaluri* outbreaks in marine aquaculture systems. Because *E. ictaruli* rarely affects marine fish, *E. tarda* is mainly reviewed in this section.

7.2. Introduction

Sakazaki (1967) studied a bacterial group, given the vernacular name "Asakusa Group", from reptilian (mainly snake) isolates. King and Adler (1964) described the "Bartholomew Group" for undescribed species of Enterobacteriaceae isolated from human diarrheal stools. Ewing *et al.* (1965) found similar biochemical characteristics of the faecal origin bacterial group of "bacterium 1483-59", the "Asakusa Group", and "Bartholomew Group", then proposed the creation a new species, *Edwardsiella tarda*.

Although *E. tarda* is the current specific epithet, Hoshina (Hoshina, 1962) isolated an enteric bacterium named *Paracolobacterium anguillimortiferum,* which is thought to be synonymous of *E. tarda*, from Japanese eel (*Anguilla japonica*) suffering "red disease" (Hoshina, 1962; Wakabayashi and Egusa, 1973). This may be the first report of *E. tarda* isolated from fish. Septicaemia of fish caused by *E. tarda* is often a chronic problem that increases not only mortality, but also production costs, reduces feed conversion, and delays harvest. To date, infections of economically important fish species with *E. tarda* have been reported throughout the world, e.g. cultured channel catfish (*Ictalurus punctatus*) in the USA, Asian (walking) catfish (*Clarias batrachus*), carp, Japanese eel, Japanese flounder (*Paralichthys olivaceus*), and red sea bream (*Pagrus major*) in Asian countries, and cultured turbot (*Scophthalmus maximus*) in Europe (Evans et al, 2011).

7.3. Disease Agent

7.3.1 Characteristics

Edwardsiella tarda is a Gram-negative, non-sporing, rod-shaped bacterium that measures 0.5 by 1.0–3.0 μm. The organism is peritrichously flagellated and motile (Ewing et al, 1965; Wakabayashi and Egusa, 1973), although non-motile (atypical) strains have been detected (Matsuyama et al, 2005). The type strain of this bacterium is ATCC 15947. *Edwardsiella tarda,* which tolerates comparatively higher NaCl concentrations than *E. ictaluri*, grows in media with 0–4% NaCl (w/v) and some strains tolerate 4.5% NaCl. This may be one of the reasons why *E. tarda* prevail in marine aquaculture fish species. *Edwardsiella tarda* grows at 15–42°C, with maximum growth at 30–37°C. It can grow in water with a pH range of 5.5 to 9.0, but optimal growth is at pH 7.5–8.0 (Wakabayashi and Egusa, 1973; Ishihara and Kusuda, 1982). The organism produces H_2S and indole

from tryptophan on triple sugar iron agar. Catalase, lysine and ornithine decarboxylase are also produced, but cytochrome oxidase and β-galactosidase are not. It does not ferment any carbohydrate or sugar alcohols except glucose, maltose and occasionally glycerol (Ewing et al, 1965; Wakabayashi and Egusa, 1973; Abbott and Janda, 2006).

7.3.2 Genome Size

Wang et al. (2009) reported the complete genome sequence of *E. tarda* EIB202 isolated from diseased turbot. *Edwardsiella tarda* EIB202 possesses a single chromosome of 3,760,463 bp containing 3,486 predicted protein coding sequences, and a 43,703 bp conjugative plasmid harboring multi-drug resistant determinants and encoding type IV A secretion system components. The sequence has an average G + C content of 59.7%.

7.3.3 Serological Classification

Two serotyping schemes for *E. tarda* had been developed independently in Japan and the USA. For international use, these schemes were merged in 1988 by Tamura et al. (1988) to establish a single serotyping scheme comprising 61 O groups and 45 H antigens. Two hundred and seventy (270) isolates of *E. tarda* collected from kidney tissues of diseased eels, rectum contents of eels, and water and sediments from eel ponds, were serologically classified into four serotypes (A, B, C and D) by Park et al. (1983). They found that 72% of the kidney isolates belonged to serotype A, whilst there were no great differences in the composition of the serotypes amongst isolates from the rectum contents, water and sediments from eel ponds. Furthermore, by experimental challenge to eels and other freshwater fish species, it was demonstrated that serotype A of *E. tarda* had a higher virulence than other serotypes. It is also reported that the majority of *E. tarda* isolated from diseased Japanese flounder was identical to serotype A (Rashid et al, 1994). Serotype A is therefore considered to be a dominant serotype of fish pathogenic *E. tarda*.

7.3.4 Molecular Classification

A PCR-based technique for the interspecific and intraspecific classification between typical *E. tarda*, atypical *E. tarda*, and *E. ictaluri* was established. Sakai et al. (2009) determined the nucleotide sequence of the upstream region of fimbrial gene clusters in typical *E. tarda*, atypical *E. tarda*, and *E. ictaluri*. Then they succeeded in designing three PCR primer sets from the respective characteristic sequences for differential detection of typical *E. tarda* as well as atypical *E. tarda* and *E. ictaluri*. A proteomic analysis identified a type III secretion system (T3SS)-associated *sseB*-like gene from the virulent *E. tarda* (Tan et al, 2002; 2005). Southern blot analysis targeting this *sseB*-like gene enabled discrimination between virulent and avirulent strains of *E. tarda* (Tan et al, 2002).

7.3.5 Pathogenesis

Numerous reports have described the isolation of *E. tarda* from marine and freshwater fish, mammals, birds, reptiles, and amphibians (Evans et al, 2011; Abbott and Janda, 2006). Amongst cultured fish species, barramundi (Asian sea bass) (*Lates calcarifer*), brook trout (*Salvelinus fontinalis*), carp (*Labeo rohita*), channel catfish, Chinook salmon

(*Oncorhynchus tshawytscha*), climbing perch (*Anabas testudineus*), common carp (*Cyprinus carpio*), crimson sea bream (*Evynnis japonica*), Europen eel (*Anguilla anguilla*), Europen sea bass, Indian carp (*Catla catla*), Japanese eel, Japanese flounder, banded knifefish (*Gymnotus carapo*), Mozambique tilapia (*Tilapia mossambicus*), Nile tilapia (*Oreochromis niloticus*), oyster toadfish (*Opsanus tau*), rainbow trout (*Oncorhynchus mykiss*), red sea bream, red tilapia tetrahybrids, sand goby (*Oxyeleotris marmoratus*), spotted snakehead (*Channa punctata*), turbot, yellowtail (*Seriola quinqueradiata*), and walking catfish have been infected (Evans et al, 2011). *Edwardsiella tarda* generally causes diseases in warm water fish, hence isolation of *E. tarda* has also been reported from numerous tropical ornamental and/or experimental aquarium fish (Evans et al, 2011) .

The intestine and abraded skin are the most likely sites for penetration of *E. tarda*. Once *E. tarda* penetrate into the host, the bacterium is recognized by phagocytic cells (e.g. neutrophils and macrophages). Phagocytic cells produce reactive oxygen species and play an important role in killing microorganisms. However, virulent *E. tarda* prevent the activation of the reactive oxygen species produced from phagocytes. Also, the virulent *E. tarda* can be more insensitive to H_2O_2 exposure than the non-virulent strain (Srinivasa Rao et al, 2001; Ishibe et al, 2008). These properties of virulent *E. tarda* enable them to multiply within the host phagocytic cells and disseminate throughout the body of the host, resulting in severe systemic infection. Some potential factors in this pathogenesis, such as bacterial superoxide dismutase, catalase and molecules related to T3SS have been reported (Tan et al, 2005; Mathew et al, 2001 ; Srinivasa Rao et al, 2003 ; Srinivasa Rao and Leung, 2003 ; Han et al, 2006; Okuda et al, 2009). Furthermore, the molecules that are involved in the iron acquisition system (siderophore), host cell adhesion and invasion, and hemolysis (hemolysin) are known to be important in the pathogenesis of *E. tarda* (Wang et al, 2009; Mathew et al, 2001; Srinivasa Rao and Leung, 2003; Chen et al, 1996; Hirono et al, 1997).

7.4. Diagnostic Methods

Isolation is achieved from the kidney, liver, and spleen by inoculation of material into brain heart infusion (BHI) agar, or trypticase soy (TS) agar. Small, round, transparent colonies develop in 48 h at 24–26°C on such media (Meyer and Bullock, 1973; Austin and Austin, 2007). *Salmonella-Shigella* (SS) agar media is also available for *E. tarda* isolation. A colony with a black center, indicating H_2S production, is observed on this media (see section 1.10, Edwardsiellosis in inland water).

7.4.1 Clinical Signs and Gross Pathology

Fish suffering *Edwardsiella* septicaemia are characterized by necrotic abscesses in the muscle that emit a putrid odour when incised, however, clinical signs of *E. tarda* infections vary between species of fish (Evans et al, 2011).

Common external signs of diseased Japanese flounder are a swollen abdomen and prolapsed rectum due to intensive ascites. Accumulation of milky or bloody ascetic fluid, abscess formation and pale coloration of the liver, swollen kidney with abscesses, and peritonitis are frequently observed in dissected diseased flounder (Miyazaki and Kaige,

1985) (Fig 7.1). Haemorrhagic ulcers on the head, especially in the opercular region, and body surface are observed in diseased red sea bream. Internal signs of *E. tarda*-infected red sea bream are formation of nodular lesions in the kidney, liver, spleen, and digestive tract and peritoneum (Miyazaki and Kaige, 1985; Kusuda et al, 1977; Yasunaga et al, 1982).

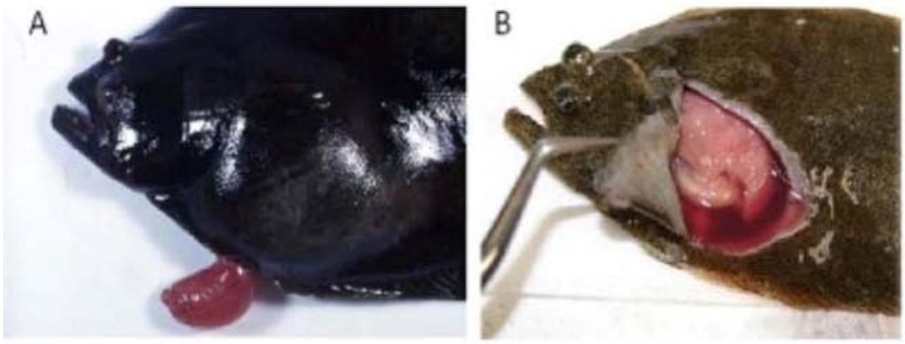

Figure 7.1. Japanese flounder infected with *Edwardsiella tarda*. A swollen abdomen and prolapsed rectum due to intensive ascites are seen frequently (A) (Photograph by Dr. Fukuda Y., Oita Prefectural Agriculture, Forestry and Fisheries Research Center). Accumulation of bloody ascetic fluid, abscess formation and pale coloration of the liver are observed when dissected (B).

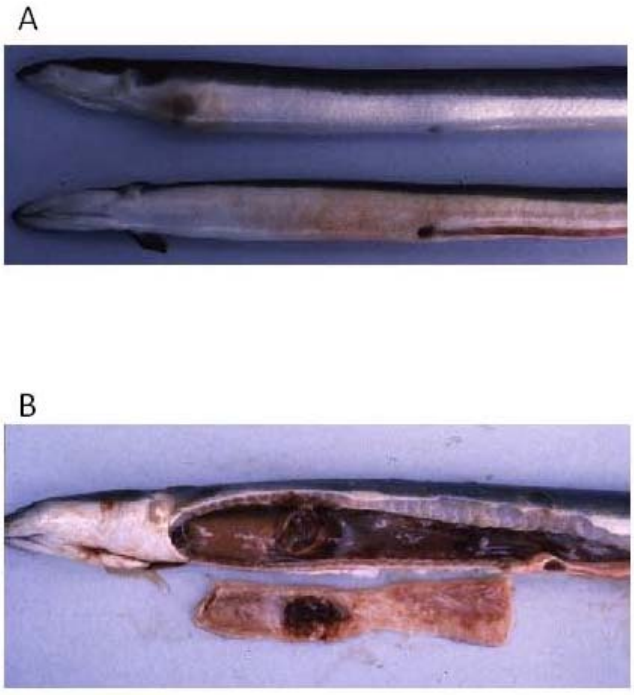

Figure 7.2. Japanese eel infected with *Edwardsiella tarda*. The upper fish has a swollen abdomen due to ulceration of the liver. The lower fish has haemorrhaging in the skin and the congested anal fin (A). The liver in diseased eel collapses due to extreme ulceration (B) (All photographs by Dr. Miyakawa M., Aichi Fisheries Research Institute).

In Japanese eel, macroscopic putrefactive lesions are characteristic. The most susceptible organ to *E. tarda* is the kidney. The affected kidney is swollen and enlarged. The ulcers in

the kidney are filled with dark red puruloid matter. The liver is occasionally infected with the bacterium, and lesions similar to those of the kidney are formed in various parts. The kidney or liver collapses and is lost in extreme cases (Egusa, 1976) (Figure 7.2). Clinical signs in tilapia are a whitish discoloration of the body surface, and a swollen abdomen due to accumulation of yellowish ascetic fluid. For internal signs, the formation of nodular lesions in the liver, spleen and kidney was reported (Miyazaki and Kaige, 1985).

The external signs of diseased channel catfish are small cutaneous lesions, each measuring 3–5 mm in diameter, located on the postero-lateral areas of the body. In acute cases, the abscesses within muscles of the flanks or caudal peduncle rapidly increase in size, and develop as large cavities filled with gas (Meyer and Bullock, 1973). Generalized congestion of internal organs and severe multifocal necrotizing inflammation in the kidney, liver, and spleen were observed in the experimentally challenged channel catfish (Egusa, 1976).

7.4.2. Histopathology

Histopathological changes of diseased fish such as ulcerative and necrotic lesions are frequently observed in the kidney, liver and spleen. Miyazaki and Kaige (Miyazaki and Kaige, 1985) described a suppurated pattern of inflammatory response in diseased Japanese eel and Japanese flounder. In such fish, formation of abscesses was seen in the kidney and liver. Observation of smeared phagocytes from the abscesses revealed their phagocytotic activities. However, the engulfed causative bacteria showed no degenerative changes and some multiplied in the cytoplasm of phagocytic cells. Similar degenerative changes were also observed in macrophages of *E. tarda*-affected turbot. This indicates the failure of macrophages to prevent the *E. tarda* infections (Padrós et al, 2006).

7.4.3. Diagnosis by PCR and Serological Techniques

A set of four primers targeting the *E. tarda* haemolysin gene was designed for the loop-mediated isothermal amplification (LAMP) method (Savan et al, 2004). This primer set allows us to detect the haemolysin gene within 45 min incubation at 65^{o}C from DNA samples of *E. tarda*-infected fish. The direct fluorescent-antibody test (DFAT) is also useful in diagnosis of *E. tarda* infection. Amandi et al. (1982) detected high numbers of *E. tarda* cells in kidney smears of diseased Chinook salmon using *E. tarda*-specific antisera.

7.5. Control

7.5.1 Prevention

Environmental stress such as high temperature, poor water quality and high organic fertility probably contribute to the onset and severity of *E. tarda*-infection. In addition, environmentally induced stress and co-infection of other bacterial pathogens are possible precursors of *E. tarda* infections. Therefore, the health management, which includes avoiding contact between pathogen and host, management of the water condition by reducing stressors (suitable oxygen concentration, low carbon dioxide and ammonia, reducing water enrichment, preventing wide temperature fluctuations), and removing

sick and dead fish as soon as possible, is important in preventing the disease (Evans et al, 2011).

7.5.2 Chemotherapy

In the USA, two antibiotics (oxytetracycline and sulfadimethoxine-ormetoprim) have been approved for *Edwardsiella* septicaemia in cultured fish. However, neither is approved specifically for *E. tarda* infections. Oxytetracycline is fed at 50 mg of drug/kg of fish/day for 12-14 days. Sulfadimethoxine-ormetoprim is fed at 50-100 mg of drug/kg of fish/day for 5 days. Withdrawal period after oxytetracycline and sulfadimethoxine-ormetoprim administration are 21 and 3 days, respectively (Evans et al, 2011; Schnick et al, 1989). Four antibiotics, florfenicol, sulfadimethoxine-ormetoprim, oxolinic acid and oxytetracycline, have been approved for *E. tarda* infection of cultured Japanese eel in Japan. Florfenicol is fed at 10 mg of drug/kg of fish/day, followed by a 7-day withdrawal period before human consumption. Sulfadimethoxine-ormetoprim is fed at 50 mg of drug/kg of fish/day, followed by a 37-day withdrawal period. Oxolinic acid is fed at 20 mg of drug/kg of fish/day, followed by a 25-day withdrawal period. Oxytetracycline is fed at 50 mg of drug/kg of fish/day, followed by a 30-day withdrawal period.

7.5.3 Vaccine

Immunization with *E. tarda* bacterins frequently shows equivocal protection, variable efficacy, short duration of protection, and lack of broad strain protection (Evans et al, 2011). Many experimental approaches have been undertaken to overcome the problems of immunization with the bacterins. *Edwardsiella tarda* ghosts are produced from the lysed bacterial cells with a lysis plasmid. Kwon et al. (2006) demonstrated high efficacy of oral immunization with *E. tarda* ghosts using Japanese flounder as a model. Kawai *et al*. (2004) showed that immunization of Japanese flounder with a conserved 37kDa outer membrane protein conferred effective protection against serologically different isolates of *E. tarda*. Recently, because of its higher efficacy, vaccination with attenuated *E. tarda* strains has attracted more attention. A single dose of the attenuated strain of *E. tarda*, which lacks the *esrB* gene, elicited significant protection against the wild-type strain of *E. tarda* (Lan et al, 2007). Takano *et al*. (2010) confirmed protective efficacy of immunization with a naturally attenuated strain of *E. tarda*, then found the up-regulated expression of interferon genes in the immunized Japanese flounder. Hence, interferon-mediated immune responses induced by attenuated *E. tarda* may be involved in protection. As described above, many studies were carried out to develop vaccines against *E. tarda*; however, commercial vaccines have not yet been approved.

7.6. Recent Topics

Wang et al. (2009) firstly determined the entire genome sequence of E. tarda EIB202, which was isolated from diseased turbot. They found numbers of aerobic or anaerobic respiration-associated genes, stress responding genes, as well as genes for signal transduction systems in the E. tarda EIB202 genome, and then surmised that E. tarda has evolved to grow and survive under diverse conditions including intracellular niches. Genes involved in the pathogenesis in a fish body including secretion systems, pili

formation, nonfimbrial adhesions, invasions and hemagglutinins, chondroitinases, hemolysins and iron scavenging systems were also identified from the genome. More recently, Yang et al. (2012) conducted comparative phylogenomic analyses of Edwardsiella species. From the genome-based phylogenetic analysis, they described two kinds of genotypes of EdwGI and EdwGII amongst six different E. tarda strains. The Edwardsiella tarda strain, which was virulent in fish, was classified into EdwGI. Surprisingly, E. tarda EdwGI strains were clustered together with the E. ictaluri lineage, and both bacterial lineages possessed highly conserved T3SS and T6SS genes, whilst avirulent E. tarda EdwGII strains lost most of the T3SS and T6SS orthologs. T3SS and T6SS of Edwardsiella species play a crucial role in the host-pathogen interaction (Tan et al, 2005; Okuda et al, 2009; Zheng and Leung, 2007; Wang et al, 2009; Rogge and Thune, 2011). Hence, it is likely that T3SS and T6SS of E. tarda EdwGI and E. ictaluri were evolutionally essential factors to adapt to their hosts. Genome-based information of Edwardsiella species will uncover the mechanisms of their pathogenicity and may facilitate the development of prophylactic and therapeutic methods.

Glossary

ATCC : American Type Culture Collection, PCR: Polymerase Chain Reaction

8. BACTERIAL HEMOLYTIC JAUNDICE

Takaji Iida

8.1. Synopsis

Bacterial hemolytic jaundice is a disease affecting cultured yellowtail, *Seriola quinqueradiata*, in Japan. Because of high total bilirubin concentrations through hemolysis by the causative bacterium, the diseased fish exhibit yellow coloration of the skin and muscle. The bacterium is a new genus and species belonging to the family Flavobacteriaceae. PCR using primers specific for this bacterium was developed for diagnosis of this disease. Chemotherapy was shown to be efficient in controlling the disease and recent results suggest that an effective vaccine is expected to be produced.

8.2. Introduction

Since 1980, a disease called “jaundice” has been known to affect cultured yellowtail, *Seriola quinqueradiata*, in Japan. This disease is prevalent during summer to autumn among mainly two year-class fish with mortality reaching up to 20%. The diseased fish exhibit yellow coloration of the skin and muscle, low hematocrit values and high total bilirubin concentrations. Initially, some factors such as environmental or nutritional stresses were proposed as the cause of this disease. In blood smears from diseased fish stained with Giemsa, thin rod-shaped organisms were found. Injection of the blood from the diseased fish to healthy individuals induced jaundice, indicating the infectious nature of the disease. An organism was isolated from the diseased fish, and injection or bath immersion using the isolate developed the jaundice in yellowtail. The isolate possessed a single cell with a cell wall and an inner membrane, without a nuclear membrane, indicating that it is a bacterium. These results confirmed that this disease was caused by bacterial infection (Sorimachi *et al.*, 1993), and later referred to as “bacterial hemolytic

jaundice" (Maeno *et al.*, 1995). To date, this disease has been reported only in cultured *S. quinqueradiata*, in Japan.

8.3. Causative Agent

The bacterium is a Gram-negative, cytochrome oxidase-positive and catalase-positive rod, 4~6 μm in length and 0.3 μm in width. The live cells of the bacterium exhibit crawling motility, but possess no flagella and pili (Sorimachi *et al.*, 1993). Culture media without FBS cannot support the growth of the bacterium. The bacterium grow in L-15 medium and Eagle MEM with FBS, with the former yielding better growth. The bacterium grow at temperatures of 20-26ºC (optimal: 23-26ºC), NaCl concentration of 0.8-3.2% (optimal: 1.6-2.0%) and pH of 6.0-8.5 (optimal: 7.0-7.5). The bacterium can be inactivated in distilled water within 3 h, and in sea water within 5 d. Survival in 1/3 sea water is longer than in 0.85% NaCl solution, and there is no difference between survival of the bacterium in 1/3, 1/2 and 1/1 sea water. The bacterium exhibited hemolytic activity against not only yellowtail red blood cells but also horse red blood cells. However, any extracellular hemolytic factors were not detected. The mechanism of the hemolysis has not been fully investigated. Although detailed studies of serotypes have not been performed, the bacterium has at least a common antigen.

8.4. Diagnostic Methods

Diseased fish display symptoms such as yellow coloration of the skin and discoloration of the gills. Yellow coloration of the muscle and enlarged spleen are commonly observed under autopsy (Figure 1). There are low hematocrit values, and high plasma hemoglobin and total bilirubin concentrations in the blood. Histopathologically, severe anemia and necrosis in the splenic hematopoietic tissues, and degeneration and necrosis in the renal tubules and hematopoietic tissues are observed. Thin rod-shaped bacteria are frequently detected in the spleen and kidney (Maeno *et al.*, 1995). The bacteria are also easily observed in blood smears stained with Giemsa (Figure 2). A set of specific PCR primers for the 16S rDNA gene of the bacterium was reported for diagnosis of this disease (Mitsui *et al.*, 2004).

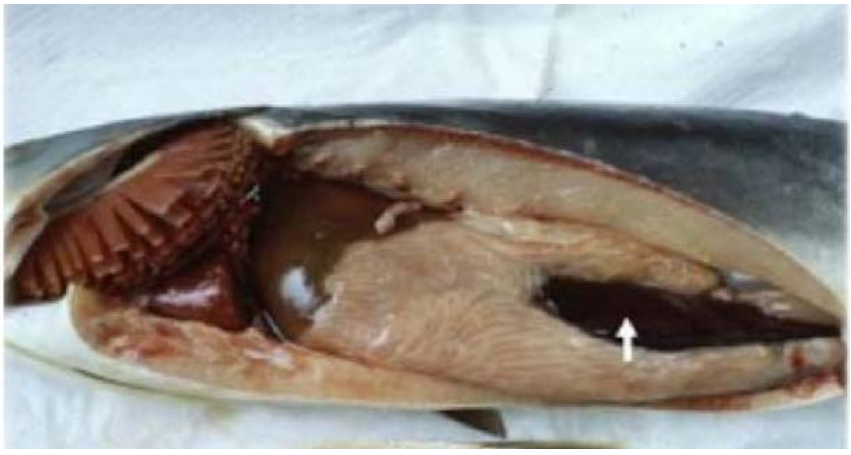

Figure 8.1. Yellowtail infected with bacterial hemolytic jaundice showing discoloration of gills and enlarged spleen (arrow).

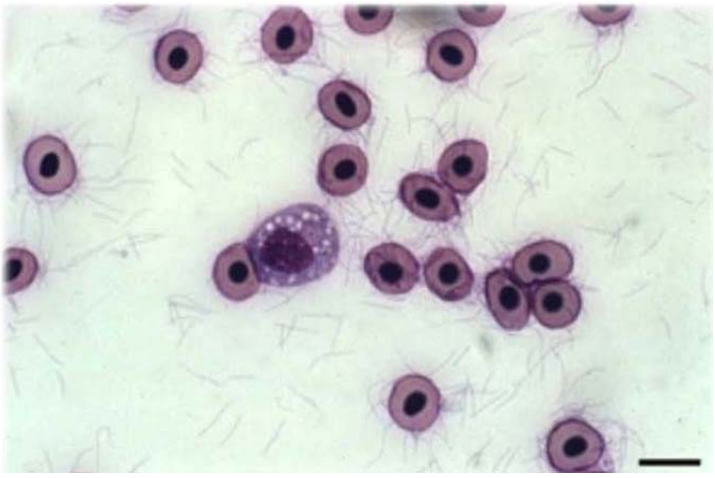

Figure 8.2. Bacterial cells in a blood smear with Giemsa stain. Scale bar: 10 um

8.5. Control

The bacterium was highly sensitive to drugs such as oxytetracyclin, tetracycline, ampicillin and erythromycin. In fish intraperitoneally injected with the bacteria and treated with various antimicrobials, more than 80% of the drug-administered group survived, while the control group only has 10% survival rate (Figure 3) (Sorimachi and Maeno, 1993). This suggests that chemotherapy is effective in controlling the disease. Fish that survived the initial artificial challenge also survived the subsequent infection challenge, and produced antibody against the bacterium (unpublished data). From these results, an effective vaccine is expected to be produced. Since mass culture of the bacterium is very difficult, genetically engineered vaccine is now being studied.

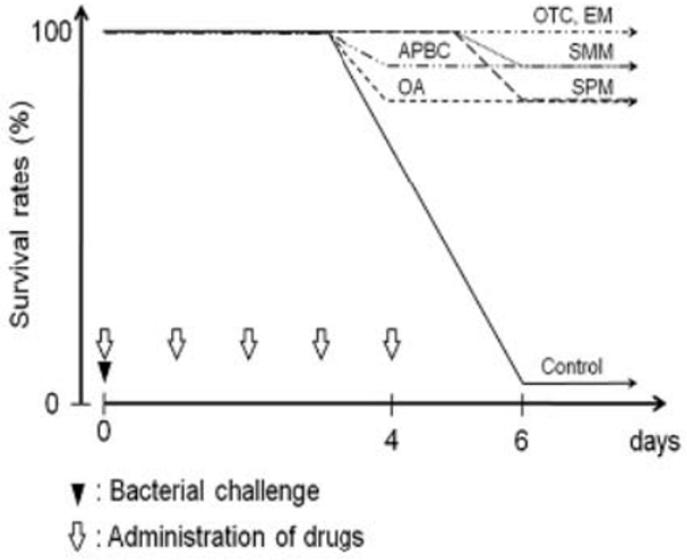

Figure 8.3. Effects of oral administration of drugs in yellowtail fry challenged with bacterial hemolytic jaundice. Abbreviation: ABPC, ampicillin; EM, erythromycin; OA, oxolinic acid; OTC, oxytetracycline; SMM, sulfamonomethoxine; SPM, streptomycin. (This figure is cited from Sorimachi and Maeno, 1993)

8.6. Recent Topics

Analysis of the 16S rDNA sequence shows that the bacterium is a member of the family Flavobacteriaceae, but does not belong to any existing genus in the family. *Icthyobacterium seriolicida* gen. nov., sp. nov. has been proposed as the name of the bacterium (in preparation).

Glossary

rDNA : Ribosomal DNA,

ABPC : Rmpicillin,

EM : Erythromycin,

OA : Oxolinic acid:

OTC : Oxytetracycline,

SMM : Sulfamonomethoxine,

SPM : Streptomycin

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Biographical Sketches

Dr. Terutoyo Yoshida is currently a professor for the University of Miyazaki, Miyazaki, Japan. He obtained Ph.D. degrees from Kagoshima University in 1991, and worked with fish bacterial diseases. He can be reached through t-yosh@cc.miyazaki-u.ac.jp

Dr. Issei Nishiki is currently a research assistant for the National Research Institute of *Fisheries Science*, Fisheries Reaserch Agency, Kanagawa, Japan. He obtained Ph.D. degrees from Miyazaki University in 2014. His main research is the fish pathogenic group C *Streptococcus dysgalactiae*.

Dr. Masahiro Sakai is currently a professor for the faculty of Agriculture, University of Miyazaki. He obtained PhD degrees from The University of Tokyo. Dr. Sakai has worked with fish immunostimulants and fish cytokines for many years.

Dr. Kim Thompson is a Senior Research Fellow at the Institute of Aquaculture, University of Stirling, UK, with over 20 years experience in fish immunology and diagnostics.

Prof. Alexandra Adams is Professor of Aquatic Immunology and Diagnostics at the Institute of Aquaculture, University of Stirling, UK and is internationally known for her work on immunodiagnostics and fish health. They have both been involved in mycobacteriosis research for over 20 years.

Dr. Tae-Sung Jung is a professor and charge of Lab. of Aquatic Animal Diseases, College of Veterinary Medicine Gyeongsang National University, South Korea. He completed his Ph. D degrees from Institute of Aquaculture, University of Stirling, UK in 1999. Dr Jung has worked in fish diseases and immunology for many years.

Dr. Takashi Aoki is currently a visiting professor for the Consolidated Research Institute for Advanced Science and Medical Care, Waseda University. He obtained Ph.D. degrees from The University of Tokyo in 1973. Dr. Aoki has worked with fish disease, chemotherapy and immunology for many years.

Dr. Yukinori Takahashi is currently a professor for the Department of Applied Aquabiology, National Fisheries University in Shimonoseki, Japan. He obtained Ph.D. degrees from The University of Kyoto. Dr. Takahashi has worked with *fish pathology* and vaccine for many years.

Dr. Jun-ichi Hikima is currently an associate professor for the Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki in Miyazaki, Japan. He obtained Ph.D. degrees from Tokyo University of Marine Science and Technology (Former name is Tokyo University of Fisheries) in 2000. Dr. Hikima's major research is infectious disease and immunity in fish.

Dr. Hisatsugu Wakabayashi is currently an Emeritus Professor of the University of Tokyo. He obtained Ph. D. degree from the University of Tokyo in 1975. Dr. Wakabayashi has worked with fish diseases, mainly focused on bacterial diseases for many years.

Dr. Tomokazu Takano is currently a researcher for Aquatic Animal Health Division, National Research Institute of Aquaculture, Fisheries Research Agency in Japan. He obtained Ph.D. degrees from Tokyo University of Fisheries in 2006. Dr. Takano has worked with fish immunology and vaccines against economically important pathogens in aquaculture for many years.

Dr. Takaji Iida is currently Director-General, Japan Sea National Fisheries Research Institute, Dr. Iida has worked with fish bacterial diseases and fish immunology for many years.