[4] Diseases Caused By Bacterial Pathogens in Saltwater

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.*

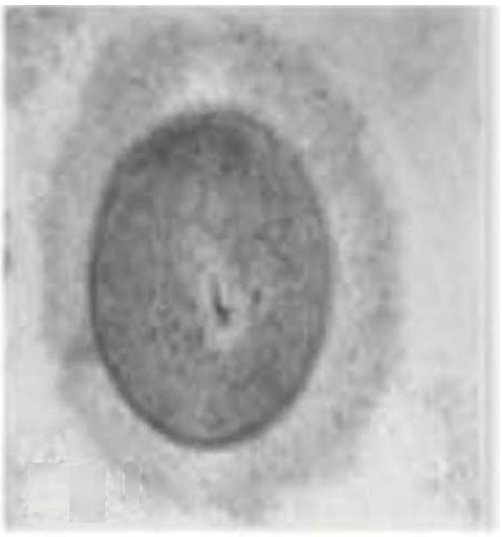

Figure 1.1.1. Capsulated virulent strain of *L. garvieae*

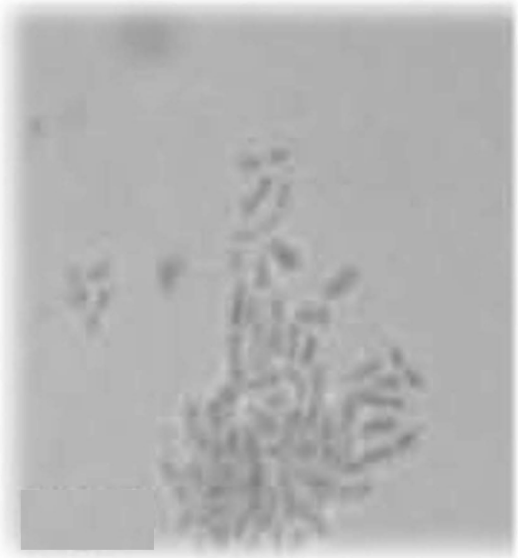

Figure 1.1.2. Phase contrast microscopy of well developed capsular strain of *L. garvieae*

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).

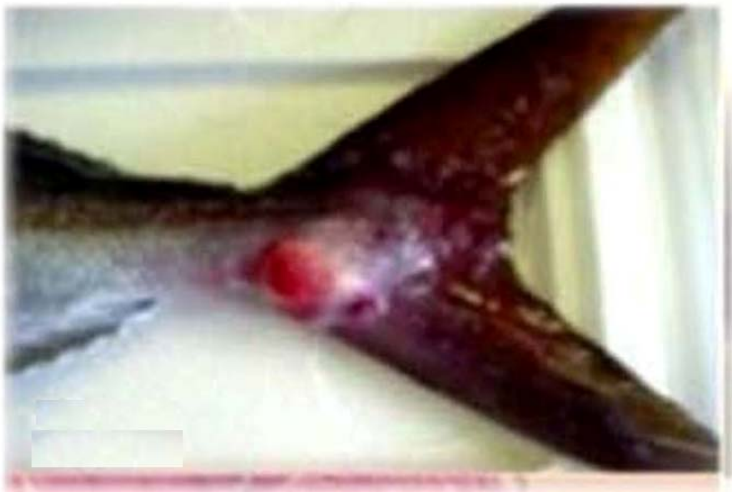

Figure 1.2.1. Severe necrosis with abscess at the caudal peduncle in infected fish

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).

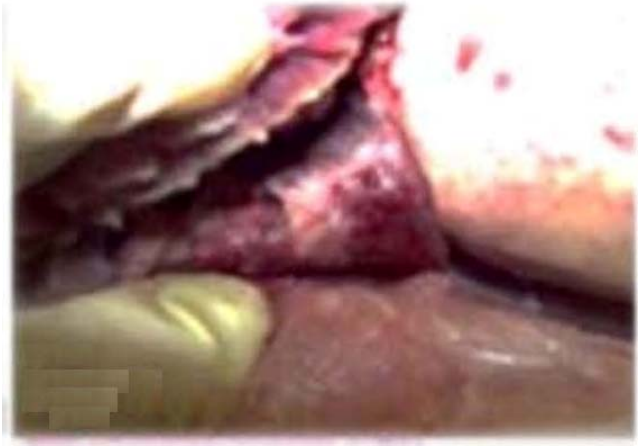

Figure 1.2.2. Severe epicarditis in infected fish

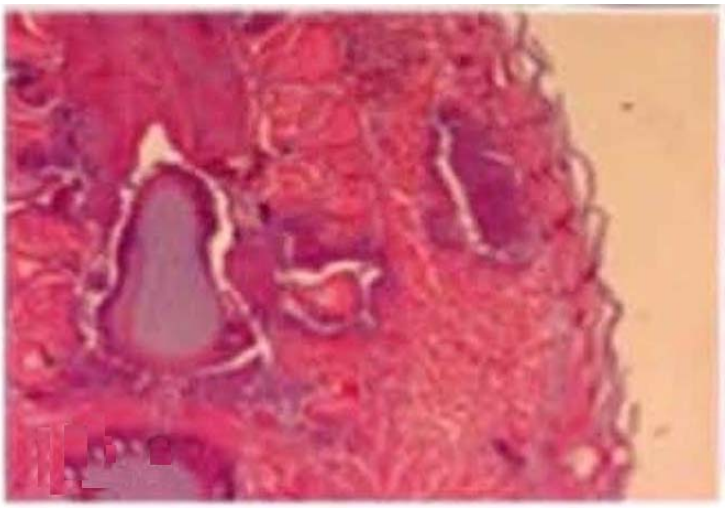

Figure 1.2.3. Histopathology of caudal peduncle in infected fish

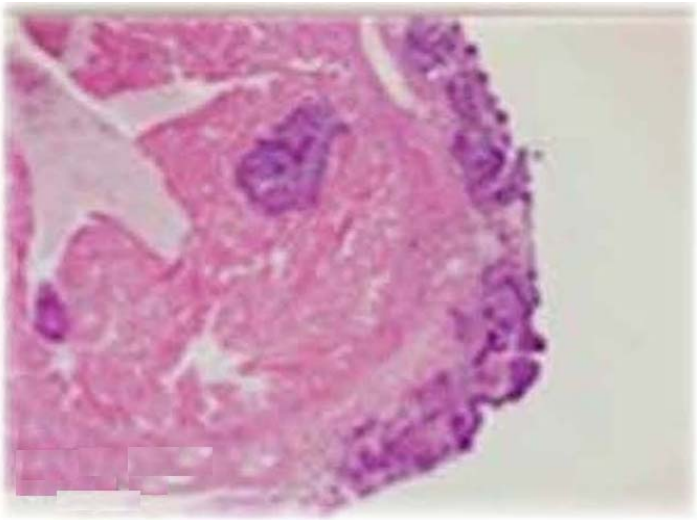

Figure 1.2.4. Histopathology of epicarditis in infected fish

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

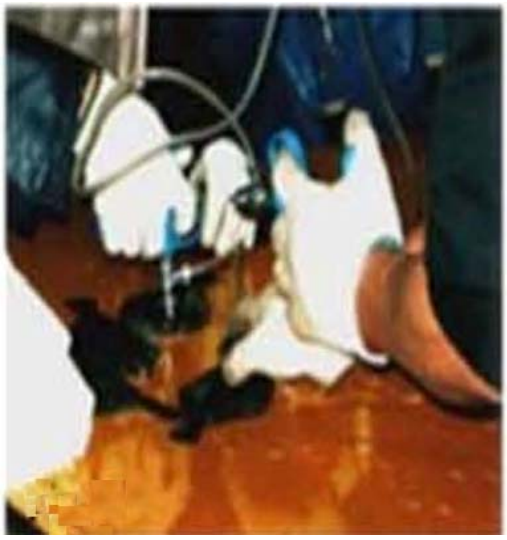

Figure 1.3.5. Injectable vaccine of *S. iniae* for Japanese flounder (Courtesy of Dr. Y. Fukuda, Fisheries Research Division, Oita Prefectural Agriculture, and Fisheries research Center, Oita, Japan)

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.

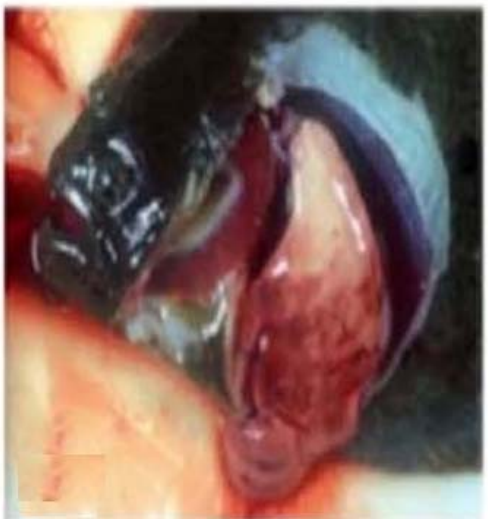

Figure 1.4.1. Infected flounder showing severe hemorrhages of liver associated with ascites (Courtesy of Dr. Y. Fukuda, Fisheries Research Division, Oita Prefectural Agriculture, and Fisheries research Center, Oita, Japan)

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*