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DISEASES CAUSED BY BACTERIAL PATHOGENS IN INLAND WATER

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Contents

Summary

Bacterial diseases cause huge damages in fish farms worldwide, and numerous bacterial pathogens from inland and saline waters have been identified and studied for their characterization, diagnosis, prevention and control. In this chapter, eight important fish diseases *viz.* 1) streptococcosis (inland water), 2) furunculosis, 3) bacterial gill disease, 4) columnaris disease, 5) bacterial cold-water disease, 6) red spot disease, 7) edwardsiellosis (*Edwardsiella ictaluri*), and 8) motile aeromonads from inland water were included covering the topics such as characteristics of disease agent, and pathogenesis, histopathological interest, diagnostic method, chemotherapy and disease control.

1. INLAND WATER STREPTOCOCCOSIS

Terutoyo Yoshida

1.1. Synopsis

Streptococcosis caused by the genera *Streptococcus* and *Lactococcus*, occurs in cultured and wild fish in freshwater, brackish water, and seawater environments due to the worldwide development of intensive fish farming practices. The genera *Streptococcus* and *Lactococcus* are facultative anaerobic, catalase-negative, and morphologically Gram-positive cocci. Historically, hemolysis on blood agar and Lancefield serological grouping have been used to identify and classify pathogenic *Streptococcus* spp. Fish pathogenic *Streptococcus* spp. and *Lactococcus* spp. are also classified into α, β, and γ (non-hemolysis) hemolysis types and Lancefield groups B, C, and non-typable.

Inland freshwater streptococcosis in cultured fish is caused by several bacterial pathogens, including *L. garvieae, L. piscium*, *S. iniae*, *Vagococcus salmoninarum,* and Lancefield serological group B *S. agalactiae* (GBS) and group C *S. dysgalctiae*. *L. garvieae*, *S. iniae,* and *S. agalactiae*, and *S. dysgalactiae* cause serious diseases in freshwater fish and in cultured and wild saltwater fish. In particular, a mass mortality of wild mullet occurred in Kuwait Bay due to β-hemolytic *S. agalactiae* infection (Evans *et al*., 2002) (Figure 1.1). Although *S. agalactiae* causes diseases in marine fish, this section focuses on Lancefield serological group B *S. agalactiae* (GBS), *L. piscium,* and *V. salmoninarum* as causal agents of streptococcosis in freshwater fish. *L. garvieae, S. iniae, and S. dysgalactiae* also cause diseases in salmonids, sweetfish (*Plecoglossus altivelis*), or tilapia in freshwater environments. These pathogens will be described in the section on bacterial pathogens in saltwater streptococcosis.

1.2. Introduction

Inland water streptococcosis occurs in freshwater fish species, mainly tilapia and salmonids. *S. agalactiae* causes diseases in warm water species including tilapia. *S. agalactiae* infections occur in cultured and wild fish species in both marine and freshwater environments in many countries including Australia, Brazil, Kuwait, Israel, USA, and Thailand. Streptococcosis in salmonids under low water temperature conditions is caused by *V. salmoninarum* and *L. piscium* infections. This section also introduces these pathogens.

Figure 1.1. *Streptococcus agalactiae* infection in wild fish in the Kuwait bay (Photo courtesy of Prof. M. Endo, Tokyo University of Marine Science and Technology)

1.3. *S. agalactiae* (= *S. difficilis*), a Disease Agent

S. agalactiae belongs to the Lancefield group B serotype. In general, Lancefield group B streptococci are synonymous name as *S. agalactiae*. Previously identified as non-hemolytic *S. difficilis* (=*S. difficile*. Eldar *et al*., 1994) and mainly isolated from tilapia, it was originally described as a non-typable Lancefield serological group. Later, *S. difficilis* (=*S. difficile*) was found to belong to Lancefield group B and capsular polysaccharide antigen type Ib (Vandamme *et al*., 1997), and this bacterial classification was proposed as a later synonym of *S. agalactiae* (Kawamura *et al*., 2005)

S. agalactiae (= *S. difficilis*) infections are found in many freshwater and marine fish species (Evans *et al*., 2002; Mian *et al*.; 2009; Geng *et al*., 2012). In particular, *S. agalactiae* exhibited high virulence during infection trials in Nile tilapia *Oreochromis niloticus*, which is an important cultured freshwater fish (Mian *et al*., 2009). *S. agalactiae* isolates from fish were positive for hippurate hydrolysis and the Voges-Proskauer reaction. The isolates were negative for the pyrolidonyl arylamidase reaction and hydrolysis of urea and starch. Acid was produced from ribose, but not from sorbitol, mannose, and xylulose. Hemolytic (beta) and non-hemolytic strains were isolated from fish (Evans *et al*., 2008). Streptococcosis in tilapia aquaculture is mainly caused by *S. agalactiae* and *S. iniae.* These strains are distributed in several countries. Table 5.1 in the section on *S. iniae Diseases caused by Bacterial Pathogens in Saltwater*: Saltwater Streptococcosis) shows the different bacteriological characteristics between *S. agalactiae* and *S. iniae*.

1.4. Diagnostic Methods

1.4.1. Serological Classification

S. agalactiae (= *S. difficilis*) from fish possess a Lancefield serological group B antigen and capsular serotype Ib antigen (Vandamme *et al*., 1997). Evans *et al* (2008) reported that fish isolates (serotype previously unreported in group B *Streptococcus*) from several countries (Kuwait, Brazil, Israel and USA) were typed as capsular serotype Ia. The ability

of BioStar STREP B Optical ImmunoAssay (STREP B OIA, a BioStar^{R}OIA^{R}Strep B assay kit; BioStar Incorporation, Louisville, CO, USA) to identify GBS isolated from aquatic animals was evaluated and found to be useful for identifying GBS strains cultured or directly collected from clinically infected fish (Evans *et al*., 2010).

1.4.2. Clinical Signs and Pathogenicity

The virulence of *S. agalactiae* isolated from fish, bovine, and humans was investigated in Nile tilapia. Some strains isolated from fish, bovine, and humans caused meningoencephalitis in the fish (Pereira *et al*., 2010). Fish infected with GBS may swim erratically and spirally, and exhibits darkened coloration. Exophthalmia and corneal opacity are typical clinical signs of infected fish. Periocular and intraocular hemorrhage was also observed in some infected fish (Bowater *et al*., 2012) (Figure 1.2). In experimental infections, septicemia with severe mononuclear infiltration was found in the meninges, epicardium, and eye (Filho *et al*., 2009).

Figure 1.2. Affected fish showing intraocular hemorrhages (Photo courtesy of Prof. M. Endo, Tokyo University of Marine Science and Technology)

1.4.3. PCR for Identification

Several PCR assays targeting species-specific regions of *S. agalactiae* 16S rRNA (Shome *et al*., 2011) for multiplex PCR assay and 23S rRNA have been developed (Kawata *et al*., 2004).

1.4.4. Molecular Classification

A high similarity was observed in whole genome DNA-DNA hybridization between *S. agalactiae* and *S. difficilis* (Kawamura *et al*., 2005). The genetic relatedness of fish, dolphin, human, and bovine GBS strains isolated from different geographical regions was examined using multilocus sequence typing (Evans *et al*., 2008). Phylogenetic trees of house-keeping genes (*gyrB*, *sodA*, *gyrA*, and *parC*) revealed that fish isolates of *S. difficilis* and *S. agalactiae* composed one cluster in which other pyrogenic *Streptococcus* species isolated from animals were not included (Kawamura *et al*., 2005).

1.4.5. Genome Analysis

Recently, *S. agalactiae* STIR-CD-17 genome was submitted to NCBI. The non-hemolytic strain was isolated from a moribund fish during a disease outbreak in farmed tilapia (*Oreochromis* sp.) in Honduras in 2008. The draft genome sequence of STIR-CD-17 has been deposited in GenBank under the accession number ALXB00000000 (Delannoy *et al*., 2012).

1.5. Control

1.5.1. Vaccine

An oil-adjuvant vaccine against *S. agalactiae* serotype II (Ib) was commercialized for tilapia and other susceptible species in Brazil and Indonesia (http://www.merk-animal-health.com/news/). A concentrated extracellular product (ECP) vaccine for *S. agalactiae* was developed for 30-g tilapia to induce antibody-mediated immunity through intraperitoneal and bath immersion administration (Evans *et al*., 2004).

1.5.2. Vagococcus Salmoninarum

V. salmoninarum infection is an emerging disease in European trout farms (Daly, 1999). The genus *Vagococcus* is motile, similar to the genus *Lactococcus*, and a new species has been proposed as *V. fluvialis* (Collins *et al*., 1989). Atypical lactobacilli isolated from diseased salmonid fish were identified as *Vagococcus* spp. Analysis of 16S rRNA sequence data clearly indicated that atypical lactobacilli isolated from fish were phylogenetically closer to the genera *Enterococcus* and *Vagococcus* than to the genus *Lactobacillus*. Atypical fish lactobacilli strains were found to belong to the genus *Vagococcus* and a new species different from *V. fluvialis* was identified as *V. salmoninarum* (Wallbanks *et al*., 1990). Wallbanks *et al*. (1990) reported a detailed description of *V. salmoninarum* isolated in USA. Schmidtke and Carson (1994) also reported on *V. salmoninarum* isolated in Tasmania, Australia, and Norway, in addition of new finding data to the report by Wallbanks *et al.* (1990).

V. salmoninarum grows at 5°C and 30°C, but not at 40°C; it produces acid from amygdalin, cellobiose, fructose, glucose, maltose, mannose, salicin, starch, sucrose, and trehalose; it produces H_2S and its G+C content was between 36.0 and 36.5 mol% (Wallbanks *et al*., 1990). Salmonid strains exhibited α-hemolysis on sheep's blood agar and grew at pH 9.6, 10□ and in 4% NaCl w/v, but not 6.5% NaCl. Lancefield serological group D and N antigens were not detected. *V. salmoninarum* could be differentiated from similar fish pathogens including *L. garvieae* (=*Enterococcus seriolicida*) and *L. piscium* (Schmidtke and Carson,1994).

1.5.3. Pathogenicity

Vagococcosis occurs at lower water temperatures (10°C−12°C) compared to other streptococcosis (Michel *et al*., 1997). *V. salmoninarum* causes chronic infections and drug treatments were ineffective in rainbow trout. Hyperemia and hemorrhage occurred in

cardiovascular lesions in the gills and viscera of infected rainbow trout (Michel *et al*., 1997).

1.5.4. L. Piscium

A lactic acid bacterium of uncertain taxonomic position isolated from diseased salmonid fish was identified as *L. piscium* (Williams *et al*., 1990). Chemical and molecular taxonomic studies such as fatty acid analysis, DNA base composition, and 16S rRNA sequencing were performed on a typical unknown lactic acid bacterium isolated from diseased salmonid fish and related bacteria including *Vagococcus* spp. (*V. fluvialis* and *V. salmoninarum*). Based on these results and detailed bacteriological charcteristics, these unknown bacteria were proposed to comprise a new species, *L. piscium* sp. nov. (Williams *et al*., 1990). Williams *et al*. (1990) reported *L. piscium* as follows: (1) its cell shape was either short rod-like or ovoid; (2) it could grow at 5°C and 30°C, but not at 40°C; (3) it could not produce H_2S; and (4) its G+C content was 38.5 mol%, as determined by melting temperature.

1.6. Recent Topics

1.6.1. Emerging Streptococcosis

Mortalities of channel catfish (*Ictalurus punctatus*) brood stock caused by unidentified streptococcal infections have been observed at several aquaculture sites in the Mississippi Delta. The main causes of mortality were arthritis, osteolysis, myosis, and spinal meningitis. DNA-DNA hybridization, 16S rRNA analysis, and other biochemical tests revealed the causative agents belonged to the genus *Streptococcus*, and a new species, *S. ictaluri*, was proposed by Shewmaker *et al*. (2007).

2. FURUNCULOSIS

Tetsuichi Nomura

2.1. Synopsis

Furunculosis, caused by the Gram-negative, non-motile, fermentative, rod-shaped bacterium, *Aeromonas salmonicida*, is one of the most serious infectious diseases of wild and farmed salmonids. The disease was first described 120 years ago from trout hatchery in Germany. Since that time, the disease and its etiological agent have been found in most salmonid hatcheries and many wild populations throughout much of the world. The pathogen can be readily isolated from kidney tissues of dead or moribund fishes using commercial media. Oral administration of antimicrobial compounds is useful for control. For prevention of this disease, vaccines are used. In spite of considerable knowledge of chemotherapy and control, furunculosis continues to be a major problem in hatcheries.

2.2. Introduction

Furunculosis, caused by the bacterial pathogen, *Aeromonas salmonicida,* is a globally important disease affecting wild and cultured stocks of salmonids and other fish species. Furunculosis was first described in the 18th century in a brown trout hatchery in Bavaria,

Germany where the manifestations of the disease included furuncule-like swellings and, at a later stage, ulcerative lesions on infected trout (Bernoth, 1997). The common name of the disease is derived from the presence of "blisters" or furuncles on the surface of chronically infected salmonids. After the initial description, numerous reports in the literature described the epizootiology and control of the disease (Schachte, 2002; Toranzo et al, 2005; Cipriano and Bullock, 2001) and the ability of both "typical" and "atypical" strains of the bacterium to cause disease (Wiklund and Dalsgaard, 1998). In spite of considerable knowledge of chemotherapy and control, furunculosis continues to be a major problem in hatcheries.

2.3. Host Range

Furunculosis occurs in many species of salmonid fish in freshwater and seawater, but the level of susceptibility is variable (Bernoth, 1997; Cipriano and Bullock, 2001). For example, among salmonids, susceptibility to infection is reported to be low in rainbow trout, while brook trout, brown trout and many other salmon species appear to have a high susceptibility. In addition, susceptibility may vary within the same fish species raised from different genetic lines, age or with different histories of exposure to the various subspecies of *A. salmonicida*. In Atlantic salmon farms, a high percentage of the fish losses are attributable to furunculosis. Spawning and smolting fish are prime victims of furunculosis due to their compromised immune status according to Department of Agriculture, Fisheries and Forestry (2009).

2.4. Disease Agent

The most important aetiological agent of furunculosis in salmonids is *Aeromonas salmonicida* subsp. *salmonicida*, which is commonly known as the "typical" strain of *A. salmonicida* and is probably the most commonly encountered bacterial pathogen in salmonids.

Characteristics: The pathogen was first described by Griffin et al. (Griffin et al, 1953). Since that time a number of subspecies of *A. salmonicida* have been recognized, although the taxonomy of the species is far from settled. Although Bergey's Manual of Systematic Bacteriology recognizes five subspecies of *A. salmonicida*: *salmonicida*, *achromogenes*, *masoucida*, *smithia*, and *pectinolytica*, many laboratories currently classify *A. salmonicida* subsp. *salmonicida* as "typical" and any isolate deviating phenotypically as "atypical". *A. salmonicida*'s ability to infect a variety of hosts, multiply, and adapt, make it a formidable pathogen (Martin-Carnahan and Joseph, 2005). *A. salmonicida subsp. salmonicida* comprises non motile, fermentative, gram-negative rods, typically 1μm x 2μm, cytochrome oxidase positive which produce a brown water-soluble pigment on tryptone-containing agar, do not grow at 37˚C, and produce catalase and oxidase. Other subspecies of *A. salmonicida* do not produce this brown pigment. Some strains of *A. salmonicida* may be cytochrome oxidase negative, a result that is inconsistent for this species. The history of the organisms reveals a plethora of synonyms including: *Bacillus devorans*, *Bacterium salmonica*, *Bacterium salmonicida*, *Bacillus truttae, Bacillus salmonicida and Hemophilus piscium* (Austin, 2011).

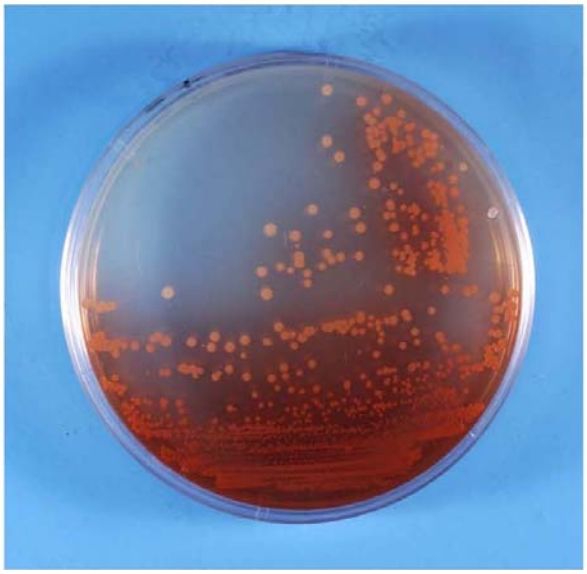

Figure 2.1. Colonies of *Aeromonas salmonicida* subsp. *salmonicida* growing on trypticase soy agar, showing water soluble brown pigment.

2.5. Genome Size

A. salmonicida subsp. *salmonicida* A449 consists of a single circular chromosome, two large plasmids and three small plasmids. The 4,702,402 bp chromosome has a G+C content of 58.5% and contains 4388 genes, with 4086 encoding proteins (Reith et al, 2008).

2.6. Serological Classification

A. salmonicida subsp. *salmonicida* can be defined as biochemically, antigenically, and genetically homogeneous with no biotypes, serotypes or genotypes being detected.

2.7. Pathogenesis

Virulence mechanisms of this pathogen fall broadly into two categories, these being cell-surface structures and extracellular products (ECPs) excreted by the cell.

Early studies of the molecular properties of *A. salmonicida* reported the presence of a special surface protein array called the A-layer or S-layer, which was responsible for the bacteria's virulent traits, and the presence of lipopolysaccharide (LPS), the cell's major cell envelope antigen. The A-layer is mainly composed of a 50Kd protein called A-protein and provides protective barrier against the defense mechanism of fish hosts. The LPS consists of three moieties; lipid A, core oligosaccharide and O-polysaccharide (o-antigen).

Since clinical signs of furunculosis are readily produced in fish injected with ECPs produced during the growth of *A. salmonicida*, an extensive body of research exists on mechanisms of virulence associated with this pathogen. The extracellular products of the pathogen consist of 25 proteins, enzymes and toxins and many more.

2.8. Diagnostic Methods

2.8.1. Clinical Signs

Furunculosis is an acute to chronic condition, with a variety of clinical signs. The disease generally appears to develop as a septicaemia and is often fatal. Affected fish often show darkening of skin, lethargy and inappetence. Haemorrhages may occur at the base of fins and the abdominal walls, heart and liver. Enlargement of the spleen and inflammation of the lower intestine are common features of chronic infections, but in acute outbreaks the fish may rapidly die without showing many signs.

Figure 2.2. Furunculosis in rainbow trout; note the furuncle cut away to show the underlying necrotic tissue.

2.8.2. Incubation Period

At 14˚C, the period from exposure of susceptible fish to this pathogen by cohabitation with infected fish to bacterial shedding can be as short as three days, death can occur as soon as two days later (i.e. at five days post-exposure). At low temperatures, the time between infection and death may be prolonged. This may be due to the effects of temperature on pathogen multiplication and host defense mechanisms (see Department of Agriculture, Fisheries and Forestry, 2009).

2.8.3. Histopathology

In sub acute/chronic infections the heart and spleen are often the most infected organs; microcolonies occur in vascular endothelia with massive destruction of spleen ellipsoids, resulting in vascular collapse; damage to spleen ellipsoids that may be accompanied by reticular cell proliferation and lymphocyte accumulation. There is degeneration of cardiac ventral epicardium and toxic cardiac necrosis, especially of the atrial lining with damage to spleen and heart.

2.8.4 Definitive Diagnosis

Presumptive diagnosis of typical *A. salmonicida* infections in salmonids is easier than the diagnosis of atypical furunculosis because clinical signs in the typical form are more stable and lesions are often not contaminated with opportunistic fungi and bacteria. Definitive diagnosis of furunculosis requires isolation and identification of the pigmented, typical strain of *A. salmonicida*. The pathogen can be readily isolated from kidney tissues of dead or moribund fishes using commercial media such as trypticase soy agar or brain-heart infusion agar plates incubated at 20-25˚C. Colonies of *A. salmonicida* subsp. *salmonicida* on these media will appear hard, friable, smooth and dark in color. After 24 hours of growth, the bacterial colonies will reach about the size of a pin point. The colonies also have a brown pigmented color that appears after they have been growing for 48-72 hours. Differentiation of colonial types that grow upon primary isolation can be facilitated by the simple addition of 0.1% (weight: volume) Coomassie Brilliant Blue (CBB) R-250 into either of the aforementioned media (CBB agar). When cultured on CBB agar, the A-layer protein that is present in virulent strains of *A. salmonicida* will absorb the CBB protein-specific dye. Consequently, virulent *A. salmonicida* develop dark blue to deep violet, friable colonies on CBB agar (Cipriano and Bullock, 2001). The API 20E rapid identification system has been widely used for identification of *A. salmonicida* subsp. *salmonicida* (Popovic et al, 2007).

2.9. Serological Identification

While cultural and biochemical characteristics produce good results, more rapid serological procedures include: serum agglutination, fluorescent antibody, or enzyme linked immune sorbent assay (ELISA) using infected tissues or isolated bacteria (Austin and Austin, 2012).

2.10. Molecular Identification

The slow growth characteristics of this bacterium, the existence of a viable, but non-culturable state, as well as the high incidence of covert infections, support the need for culture-independent, molecular diagnostic protocols (Gustafson et al, 1992). Using PCR and a specific DNA probe, the existence of *A. salmonicida* was reported in effluent, water, faces and sediment from fresh water Atlantic salmon farm. Although the highest specificity in the detection of *A. salmonicida* is obtained when the PCR assay is directed to the amplification of the surface A-layer gene, recent studies allowed the design of new primer sets targeted to the gene *fst*A (coding for an outer membrane siderophore-receptor), which showed a total specificity for *A. salmonicida* isolates (Beaz-Hidalgo and Figueras, 2012).

2.11. Control

Control methods for this disease have involved use of good husbandry practices (including good water quality, adequate disinfection of equipment and eggs, and lower stocking densities), disease-resistant fish stocks, improved diets, nonspecific immune stimulants, antimicrobial compounds, probiotics (micro-organisms that exert a beneficial effect on the host) and vaccines.

In the laboratory, typical *A. salmonicida* can be shown to be sensitive to a wide range of antimicrobial chemotherapeutants. Oral administration of these antimicrobial compounds is useful for control. Terramycin (oxytetracycline) should be added to feed at the rate of 3.0 g/100 lb fish, administered daily for 10 days to affected fish. Sulfamerazine should be administered at the rate of 5-10 g/100 lb fish and fed for 10 or 15 consecutive days (Schachte, 2002).

2.12. Prevention

Regular monitoring programs that detect *A. salmonicida* in the water supply and provide early non-lethal detection in mucus can be coupled with topical disinfection or antibiotic regimens that either preclude or minimize infection.

If eggs must be imported from outside of the hatchery system, insist that only eggs supplied from inspected and certified furunculosis-free sources be used. United States Fish and Wildlife Service regulations recommend that eggs should be disinfected by submersion for PVP-iodine after fertilization. If eggs are then shipped to another facility for incubation, policy requires that those eggs undergo a secondary disinfection using the same agent (USFWS, 1995). In practice, conduct of the stress induced furunculosis assay and regulatory confinement of infected smolts have reduced the number of furunculosis outbreaks associated with early marine culture.

2.13. Vaccine

Vaccination leads to the production of antibodies against both cellular and soluble antigens of *A. salmonicida*. Vaccination also stimulates cellular immunity. Most vaccines use oil-based adjuvants because they confer superior protection and duration of immunity compared to other adjuvants. The best results in terms of protection have been reported in salmonids with the mineral oil-adjuvanted vaccines; however, these bacterins posses several adverse side-effects. To avoid these drawbacks, new non-mineral oil-adjuvanted vaccines have been recently developed. However, recombinant DNA technology allowed the construction of highly attenuated and stable aroA auxotrophic mutant strains (using an allelic replacement technique), which were employed experimentally as safe live vaccines with high success. Approval of this method for field use has not yet been given (Press et al, 1996).

2.14. Recent topics

Although biotechnology is employed in the detection and control of furunculosis, there is still need for rapid, reliable and easy diagnostic systems suitable for covert carrier fish and field use (Austin, 1997). There is evidence to suggest that distribution and transmission of many pathogenic bacteria will increase with global warming. Few studies have tried to predict the impact of this phenomenon on *A, salmonicida* (Tam et al, 2011). Advancement in disease control measures is expected, particularly the use of probiotics, non specific immunostimulants such asβ-1, 3-Glucans and oral vaccines.

Glossary

LPS: Lipopolysaccharide
ECPs: Extracellular products
CBB: sCoomassie brilliant blue

3. BACTERIAL GILL DISEASE

Hisatsugu Wakabayashi

3.1. Synopsis

Bacterial gill disease (BGD) caused by *Flavobacterium branchiophilum* has been reported from various cultured freshwater fish species, in particular salmonids, worldwide. The bacterium is Gram-negative slender rods measuring 0.5 × 5-8 μm that are non-motile and showed neither gliding movement nor swarming growth on cytohaga agar (CA). When an outbreak of BGD occurs, *F. branchiophilum* first appears abundantly on the surfaces of the gills. A proliferative hyperplasia develops in the epithelium and progresses to clubbing and fusion of gill lamellae. Several chemical disinfectants including sodium chloride have been used to treat BGD. When the bacteria on the gills are removed by treatment at the early stage of infection, fish recover rapidly.

3.2. Introduction

Bacterial gill disease (BGD) is characterized by the presence of numerous filamentous bacteria on the surface of the gill epithelium. Davis (1926, 1927) first observed it in fry and fingerling brook trout (*Salvelinus fontinalis)* and rainbow trout (*Oncorhyncus mykiss*) in hatcheries in Vermont, USA. He called the condition bacterial gill disease, but did not attempt to isolate or identify the bacteria. Rucker et al. (1952) and Bullock (1972) isolated several strains of yellow-pigmented bacteria, referred to myxobacteria from infected gill tissue but none could be shown to be the causative agent. Kimura et al. (1978) and Wakabayashi et al. (1980) isolated a different yellow-pigmented bacterium from gill lesions of several species of salmonids in Japan and USA, and experimentally produced BGD with this organism. The bacterium was named *Flavobacterium branchiophila* by Wakabayashi et al. (1989). Later, this name was corrected to *F. branchiophilum* by von Graevenitz, (1990). BGD with *F. branchiophilum* has been reported from various cultured freshwater fish species, especially salmonids, in Japan, USA, Hungary (Farkas 1985), Canada (Ferguson et al. 1991, and Korea (Ko and Heo 1997). Probably the cause of most BGD outbreaks in salmonids is *F. branchiophilum* (Bullock 1990, Turnbull 1993). However, similar pathology could result from the presence of a low grade opportunist pathogen in extreme environmental conditions, or the presence of a highly pathogenic bacterium in marginal environmental conditions (Turnbull 1993).

3.3. Disease Agent

3.3.1. Flabobacterium Branchiophilum

Cells of *F. branchiophilum* from cytophaga broth (CB) (Anacker and Ordal 1959) are Gram-negative slender rods measuring 0.5 × 5-8 μm and usually occurred in chains of

two or three cells (Figure 3.1). They are non-motile and showed neither gliding movement nor swarming growth on cytohaga agar (CA). Growth on CA is slow and colonies are rarely visible in less than 2 day incubation. They are light yellow, round, transparent, smooth and 0.5 – 1 nm in diameter after incubation for 5 days at 18°C. The bacteria grow well at 10 to 25°C and in the presence of 0.25% NaCl or in the absence of NaCl. Some strains are also able to grow at temperatures as low as 5°C and as high as 30°C and in the presence of higher concentrations of NaCl (0.05 to 0.1%).

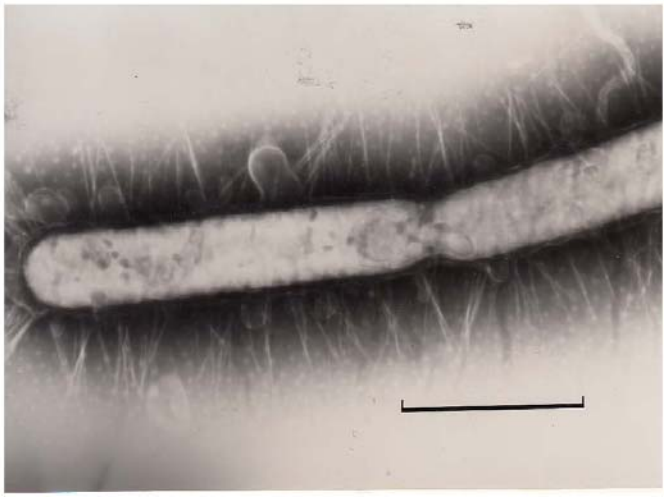

Figure 3.1. Electron micrograph of negative stained cells of Flavobacterium branchiophilum ATCC35035^T. Bar – 1 μm.

Wakabayashi et al (1989) described the biochemical characteristics of 16 strains from Japan, USA and Hungary as follows. All strains produced catalase and cyctochrome oxidase, and hydrolyzed gelatin, casein, and starch. They were negative for production of hydrogen sulfide and indole, reduction of nitrate, and degradation of chitin. Some of the strains tested were unable to grow in test medium for nitrate reduction. Also, there was no growth on DNase medium. In the cellulose digestion test, the bacteria grew on the surface of the filter paper, but disintegration of the strip did not occur. Acid but no gas was produced from plucose, fructose, sucrose, maltose, trehalose, cellobiose, melibiose, raffinose, and inulin, but not from galactose, lactose, arabinose, xylose, rhamnose, adonitol, mannitol, dulcitol, sorbitol, inositol, or salicin. The DNA G+C contents of three of the strain ranged from 29 to 31 mol% (mean, 30%). These characteristics accord with those of *F. branchiophilum* strains from Canadian salmonids with BGD (Ostland et al. 1994). However, Bernardet et al (1996) reported a G+C content range of 33-34 mol%.
The strains isolated in Japan, USA, Canada, and Hungary possessed common antigens detectable by agglutination and precipitation tests (Wakabayashi et al. 1980, Huh and Wakabayashi 1989, Ostland et al. 1994). However, the Japanese strains were distinguished from USA and Hungarian strains by agglutinin-adsorption tests, and the precipitation tests revealed one antigen specific for Japanese strains and two antigens specific for both USA and Hungarian strains (Huh and Wakabayashi 1989).

Negative-stained preparation of *F. branchiophilum* cells demonstrated fimbriae-like appendages extending from their surface (Wakabayashi et al. 1989, Ostland et al. 1994).

Ototake and Wakabayashi (1985) found that the infectivity of the bacteria was significantly reduced by a mechanical agitation of the bacterial suspension with a blender. The adherence of the bacteria to the gill tissue surface was thought to be mediated by fimbriae (pili), but the hemagglutins contained in the supernatant of the agitated bacterial suspension were non-fimbrial agglutinin (Ototake and Wakabayashi 1985). Heo et al. (1990) tried to purify and characterize the fimbriae of *F. branciophilum* ATCC35035^T. The fimbriae mechanically detached from the bacterial cells were purified by using ion-exchange chromatography on DEAE-cellulose. A main purified fimbrial subunit had a molecular weight of 23,000 dalton and a maximum absorption at 276 nm in ultraviolet absorption spectrum. In serological analysis, four strains of *F. branchiophilum* produced a single common precipitin line against rabbit anti-fimbriae serum. Although a significant reduction in the attachment of *F. branchiophilum* strains was not achieved after immersion of fish in the crude fimbrial extracts (CFEs), it was suggested that inhibition by CFE was dose dependent (Ostland et al. 1997).

In virulence studies with eight selected strains of *F. branchiophilum* strains, Ostland et al. (1995) found that all of the strains were fimbriated and adhered to the gills of rainbow trout, but that only five strains succeeded in proliferating and further colonizing the gills to cause mortality. They suggested that an additional virulence factor(s) might facilitate bacterial proliferation resulting in further branchial colonization, to induce mortality. Kudo and Kimura (1983c), and Ototake and Wakabayashi (1985) reported that *F. branchiophilum* produced an extracellular hyperplasia inducing factor which could reproduce lesions histopathologically similar to those in BGD.

3.4. Other Bacteria Associated with BGD

Thunbull (1993) summarized phenotypic characteristics of bacteria recovered from BGD. In addition, Ostland et al. (1999) reported a new form of BGD affecting intensively reared salmonids in Ontario Canada. The outbreaks occurred at water temperature less than 10 ℃. Shorten and somewhat stubby lamellae covered with swollen epithelial cells occurred in the sequel to infection, while overt epithelial hyperplasia, lamellar fusion and filamental clubbing were not common. The predominant bacterium recovered from affected gills was a short, Gram-negative rods which shared phenotypic characteristics with *Pseudomonas fluorescens*. An attempt to reproduce using the isolates was unsuccessful.

3.5. Diagnostic Methods

Diseased fish become suddenly lethargic and anorexic. They remain near the water surface or gather at the inflow site. Opercula of these fish are bilaterally flared with an irregularly eroded margin. Respiratory rates are markedly increased, and some fish exhibit labored and accentuated use of the buccal and opercular pumps (Spear and Ferguson 1989). A proliferative hyperplasia develops in the epithelium of the gill lamellae. As the disease progresses, the epithelium proliferates, causing clubbing and fusing of gill lamellae (Bullock 1990). The hyperplasia is usually seen first at the distal tips of the lamellae. Bacterial colonization begins at these lamellar tips before spreading proximally (Wood and Yasutake 1957, Kudo and Kimura 1983a, Spears et al. 1991a). The relationship between the severity of lamellar lesions and the abundance of bacteria is not

always clear since, in some cases, the bacteria are more numerous in less severely affected areas of the gills (Daoust and Ferguson 1983). However, Ostland et al. (1990) reported that statistical analysis on the severity of lesions and bacterial recovery indicated a strong association between the severity of lesions and the presence of filamentous bacteria.

Wakabayashi et al. (1980) made a scanning electron microscopic observation of the gill filaments following bath challenge of juvenile rainbow trout with pure cultures of *F. branchiophilum*. All of the cultures were able to establish abundant growth on the surface of the tissues within 18 to 24 hours (Figure3.2 and 3.3). The gill epithelium assumed hyperplastic appearance 4 days after exposure, though the bacterial cells decrease in number on the surface of the gill filaments. The general pathology of BGD is most likely caused by restrictions on the respiratory and excretory functions of the gills (Snieszko 1981). The oxygen response thresholds for fish experimentally infected with *F. branchiophilum* were 1.5 – 2.5 times higher than those of uninfected fish, and their respiratory functions seemed to be impaired 2 days after infection and became worse after 5 days (Wakabayashi and Iwado 1985a). Changes in glycogen, pyruvate and lactate concentrations in the muscle tissue of juvenile rainbow trout with BGD suggested that a breakdown in gas exchange at the gills caused the failure in circulation to provide oxygen enough to remove excess lactate from the muscle, even though the level of muscle lactate was not so high as that of healthy fish (Wakabayashi and Iwado 1985b).

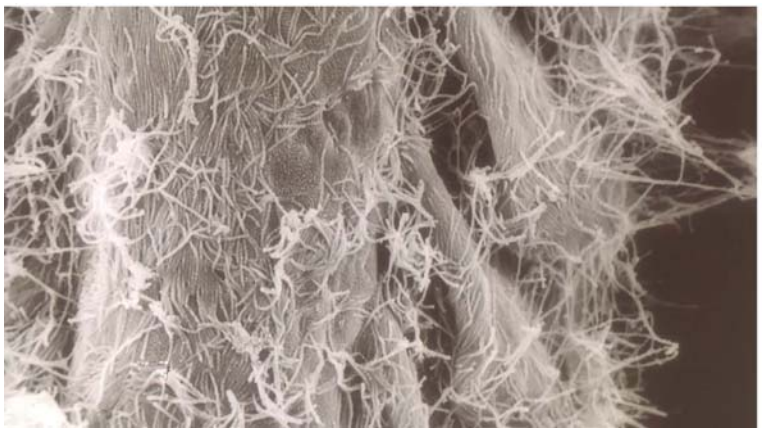

Figure 3.2. Scanning electron micrograph of *Flavobacterium branciophilum* and lamellae 18 hours after exposure of a rainbow trout fingerling to the bacterial suspension in an aquarium.

Because outbreaks of BGD occur suddenly, the disease usually cannot be diagnosed until mortalities begin. Diagnosis is based on the clinical signs along with the examination of wet mounts of the gill tissue for hyperplasia and the presence of filamentous bacteria. Phase contrast microscopy is recommended for enhanced observation of wet mount gill filaments. The causative agent is identified as *F. branchiophilum* by isolation and application of definitive biochemical tests. But *F. branchiophilum* is usually not isolated from internal organs.

A fluorescent antibody test (FAT) was used to detect from infected fish and their environment in a trout hatchery (Huh and Wakabayashi 1987, Heo et al. 1990)). An

enzyme-linked immunosorbent assay (ELISA) was developed to estimate the quantity of *F. branchiophilum* in crude gill extracts from rainbow trout following bath exposure to the bacterium (MacPhee et al. 1995a, Ostland et al. 1995). Toyama et al. (1996) reported that the PCR with a pair of a specific primer BRA1 and a universal primer 1500R succeeded in specifically amplifying 16S rDNA from *F. branchiophilum*.

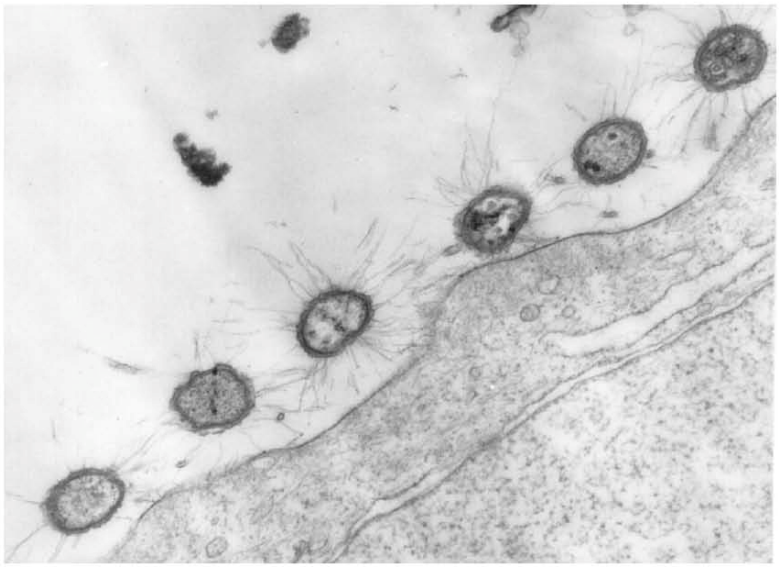

Figure 3.3. Transmitting electron micrograph of *Flavobacterium branchiophilum* cells on the gill epithelium 18 hours after exposure of a rainbow trout fingerling to the bacterial suspension in an aquarium.

3.6. Control

According to Bullock (1990), several chemical disinfectants have been used to treat BGD in salmonid hatcheries in the USA. The most widely used are quarterly ammonium compounds, such as benzalkonium chlorides. Another chemical is the herbicide Diquat. However, none of these chemicals are approved by the U. S. Food and Drug Administration for disease control in food fishes. Efforts are under way to have chloramine-T registered as a treatment for BGD (Bullock et al. 1991, Bowker and Garty 2008, 2011). Hydrogen peroxide (H_2O_2) represents a more environmentally friendly alternative because of its low regulatory priority (Lusmsden et al. 1998, Derksen et al. 1999). Rach et al. (2000) reported that two static bath treatment regimens were effective in the control of BGD: H_2O_2 administered at concentrations of 56 – 110 mg/L as a 60 min exposure or H_2O_2 administered at a concentration of 56 – 230 mg/L as a 30 min exposure. Sodium chloride (NaCl) is widely employed to treat BGD in hatchery salmonids in Japan: NaCl is used at a concentration of 1 – 5 % as a 1 – 2 min bath. Kudo and Kimura (1983a, b) demonstrated that this treatment was very effective for the removal of bacterial cells and subsequent recovery.

3.7. Recent Topics

Touchon et al. (2011) reported the complete genome sequence of *F. branciophilum* strain

FL-15 isolated from a diseased sheatfish (*Silurus glanis*) in Hungary. The FL-15 genome encodes 20 predicted adhesion precursors that could be implicated in cell-cell and cell-surface interactions. However, no genes encoding known pilus or fimbrial proteins were identified in the FL-15 genome. The authors mentioned that, if present, the corresponding genes may be hidden within those encoding hypothetical proteins; it is also possible that production of pilus-like structures is a strain-dependent feature absent from strain FL-15. It is also interesting that the FL-15 genome contains two distinct groups of genes, *gld* and *spr*, which are involved in gliding motility. This suggests that *F. branchiophilum* may actually be motile, but experimental conditions used so far failed to mimic natural conditions where gliding motility is expressed.

4. COLUMNARIS DISEASE

Hisatsugu Wakabayashi

4.1. Synopsis

Columnaris disease, caused by *Flavobacterium columnare,* affects cultured, ornamental and wild-fish populations in freshwater worldwide. The disease is characterized by external infections in the fish body surface, gills or fins. The bacterial cells are long, flexible, Gram-negative rods that are motile by gliding. They grow well on low nutrient media producing pale yellow rizoid colonies. Traditional culture techniques require several days for a definitive diagnosis. Therefore, molecular techniques such as the polymerase chain reaction (PCR) have been used. Because *F. columnare* primarily attacks gills, skin and fins of fish, most treatments proposed for columnaris disease are surface-acting disinfectants used as baths.

4.2. Introduction

Columnaris disease was first described by Davis (1922), who observed it in warm-water fish from the Missisippi River, USA. Although Davis did not succeed in isolating the pathogen, he named it *Bacillus columnaris* because, when pieces of infected tissues from diseased fish were examined microscopically in wet mount preparation, column-like masses of the bacteria were observed along the periphery of the tissues (Figure 4.1).

The etiological agent was first isolated by Ordal and Rucker (1944) from hatchery reared sockeye salmon (*Oncorhynchus nerka*) in the summer of 1943 and renamed *Chondrococcus columnaris.* At the same time Garnjobst (1945) isolated the organism from some warm water fishes and assigned it to the genus *Cytophaga*. The precise taxonomy of the agent has been the subject of continuing discussion (Leadbetter 1974, Reichenbach 1989). This taxonomical confusion was resolved when Bernardet *et al.* (1996) transferred the bacterium to the genus *Flavobacterium* (Bernardet and Bowman 2011).

Flavobactrium columnare, the causative agent of columnaris disease, is ubiquitous in freshwater environments and an opportunistic pathogen that causes skin and gill infections in freshwater fishes worldwide (Nigrelli and Hutner 1945, Wakabayashi and Egusa 1966, Anderson and Conroy 1969, Bowser 1973, Wobeser and Atton 1973, Bootsma and Clerx 1976, Fergson 1977, Morrison et al. 1981, Chun et al. 1985,

Bernardet 1989, Berno 1989, Koski et al. 1993, Figueiredo et al. 2005, Welker et al. 2005, Tien et al. 2012).

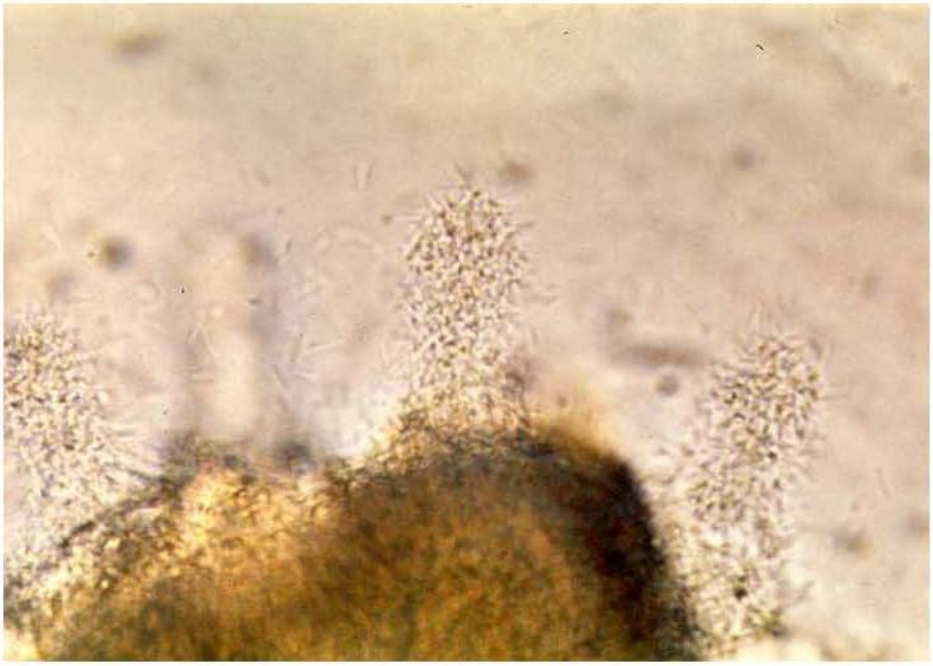

Figure 4.1. Columnar formations of *F. columnare* along the margin of a piece of the tissue.

4.3. Disease Agent

Cytophaga agar (CA) (Anacker and Ordal 1959a) is the most frequently used medium for cultivation of *F. columnare.* It contains trypton 0.05%, yeast extract 0.05%, sodium acetate 0.02%, beef extract 0.02% and agar 0.9% and is adjusted to pH7.2-7.4. The colonies are of a spreading nature with irregular margins and they adhere to the agar. Under the microscope (×40), the edge of the colonies appeared rhizoid (Figure 4.2). *F. columnare* contains flexirubin type pigments, so that the colonies change their color from yellow to brown when flooded with 20% KOH solution. In static culture in cytophaga broth (CB) the bacteria form clusters or a pellicle of cells on the surface of the broth. When gently agitated, however, they usually grow homogeneously.

The cells are Gram-negative, slender and rather long bacilli, 0.3 - 0.5 μm wide and 3 - 8μm. The filamentous cells display an active flexing movement in wet mount preparation. They have no flagella but exhibit gliding motility on a wet surface. Bernaldet and Grimont (1989) described the physiological characteristics of eight strains of *F. columnare* isolated Europe, USA and Japan as follows. Growth occurs in CB supplemented with 0.1% or 0.5% NaCl and at 10-33 C. Catalase and cytochrome oxidase are produced; nitrate is reduced to nitrite; hydrogen sulfide is produced. Cellulose, carboxymethyl cellulose, chitin, starch, aesculin and agar are not hydrolysed. No acid is produced from carbohydrates in ammonium salt-sugar medium. Gelatin, casein (skim milk agar), and tyrosine are hydrolysed. Lysine, arginine and ornithine are not decarboxylated. Tributyrin, lecithin (egg yolk), Tween 20, Tween 80 and DNA are

hydrolysed. These characteristics accord with those reported by other workers such as Garnjobst (1945), Wakabayashi et al (1970) and Bootsma and Clerx (1976).

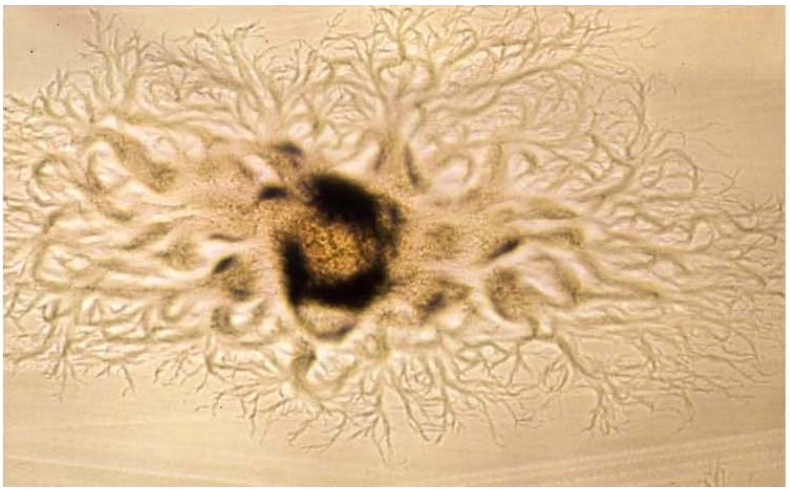

Figure 4.2. A colony of *F. columnare* growing on cytophaga agar, showing the rhizoid edges to the colony.

The DNA base composition has been variously defined as between 29.8 and 35.9 mol % G+C (Mitchell), or 32.6 and 42.9 mol % G+C (Bootsma and Clerx 1976). According to Bernardet and Grimont (1989) the base composition of their strains were 32.0 and 33.2 mol % G+C and the relatedness in DNA-DNA hybridization was more than 76%. Song et al. (1988) compared the isolates from Western North America and other areas of the Pacific Rim. They reported that the mol % G+C of the DNA was 29.6 - 32.5 and that 19 of the 22 strains were 81-98% homologous with their type strain isolated from fish in Oregon, USA. Triyanto and Wakabayashi (1999a,b) described three genomovars among the species based on the analysis of the 16S rDNA gene restriction fragment length polymorphisms (16S rDNA-RFLP). The 16S rDNA sequence provided enough variability to defined three genomovars within the species. Genomovar I is defined by the type strain IAM14301^T (=ATCC23463^T) isolated from Chinook salmon in USA and including 10 Japanese and 8 French strains from various fishes, a Chinese strain from grass carp (*Ctenopharyngodon idella*), and an Indonesian strain from common carp (*Cyprinus carpio*). Genomovar II is defined by EK28 (=IAM14820) isolated from eel (Anguilla japonica) in Japan and including a Japanese strain from loach (*Misgurnus anguillicaudatus*). Genomovar III is defined by a single strain PH97028 (=IAM14821) isolated from ayu (*Plecoglossus altivelis*) in Japan. Michel et al (2002) found that two neon tetra (*Paracheirodon innesi*) isolates and two blackmolly (*Poecilia sphenops*) isolates belonged to genomovar II, while all North American and French fresh water fish isolates belonged to genomovar I. They thought that genomovar II or Asian type strains might have been brought to Europe through ornamental fish imports. Two isolates from warm water fishes, i.e. catfish (*Ictalurus* sp.) and baitfish (*Notropis* sp.) in USA were added to the original Genomovar III isolate PH97028 (Scheck and Caslake 2006). Amplified fragment length polymorphism (AFLP) fingerprinting further subdivided the species without losing genetic hierarchy of genomovar division (Arias et al. 2004). Olivares-Fuster et al. (2007) reported that both the 16S - single strand conformation polymorphism (16S-SSCP) and the intergenic spacer region - single strand conformation

polymorphism (ISR-SSCP) improved resolution when compared with standard RFLP. The SSCP analysis of rRNA genes proved to be a simple, rapid, and most effective method for routine fingerprinting of *F. columnare* (Olivares-Fuster et al. 2007).

Anacker and Ordal (1959b) reported that the strains isolated from fish in the USA possessed a common species specific antigen and several other antigens. On the basis of their antigenic composition, strains were separated into four serotypes and one miscellaneous group. However, no correlation between serotype, geographical origin, or species of host-fish and virulence was found.

The difference in virulence among *F. columnre* strains was reported on the basis of experimental infection (Pacha and Ordal 1963). Some authors have suggested that one of the virulence factors of *F. columnre* is the extracellular proteases produced by the bacterium (Griffin 1991, Bertolini and Rohovec 1992, Teska 1993, Newton et al. 1997). Chondroitin lyase activity was found to be significantly related to the virulence of eight *F. columnare* strains (Suomalainen et al. 2006). Zhang et al. (2006) compared lipopolysaccharide (LPS) and total protein profiles from four *F. columnare* isolates and reported that it was possible to discriminate the attenuated mutant FC-RR strain from other virulent strains. Some researchers demonstrated the relationship between adhesion of *F. columnare* and virulence (Decostere et al. 1999ab, Olivares-Fuster et al. 2011), while others found no such association (Suemalainen et al. 2006, Kunttu et al. 2009).

4.4. Diagnostic Method

The first indication of the infection is generally the appearance of a white spot on some part of the head, gills, fins or body. This is usually surrounded by a zone with a distinct reddish tinge, leading to under-running of adjacent skin. Lesions on the gills or fins extend principally from the distal end towards the base, and the tissues are eroded and destroyed (Figure 4.3). Lesions are covered with a yellowish white mucoid exudate consisting largely of swarms of *F. columnare*. The bacteria are not usually found systemically until a relatively large amount of external skin or gill damage has taken place; thus it would appear that the bacteria enter the blood stream through the external lesions and are probably not directly involved in causing death (Wood 1979).

High water temperature enhances the outbreaks of columnaris disease (Fish and Rucker 1943, Ordal and Rucker 1944, Holt et al. 1975, Decostere et al. 1999, Suomalainen et al. 2005a). Wakabayashi and Egusa (1972) studied the effect of water temperature on columnaris disease in loach. Fish were challenged by immersion in water containing *F. columnare* at about 10^6 cfu/ml, then held at temperatures ranging from 5 to 35 ^oC in 5 ^oC intervals. No mortalities occurred in fish held 5 or 10 ^oC, twenty five percent of those held at 15 C died and all of the exposed fish held at 20 - 35 ^oC died. The mean times to death were 7.0, 3.0, 1.8, 1.0 and 1.0 days at 15, 20, 25, 30 and 35 ^oC, respectively. During the period 1955 – 9, the incidence of columnaris disease in salmon, especially sockeye salmon, in the Columbia River Basin, increased with increasing water temperature (Pacha and Ordal 1970). Field surveys of Fraser River spawning areas in 1963, 1964, and 1965 revealed that pre-spawning losses of sockeye salmon by columnaris disease in 1964 were comparatively very small (less than 5%) owing to the lower temperature in that year (Colgrove and Wood 1966).

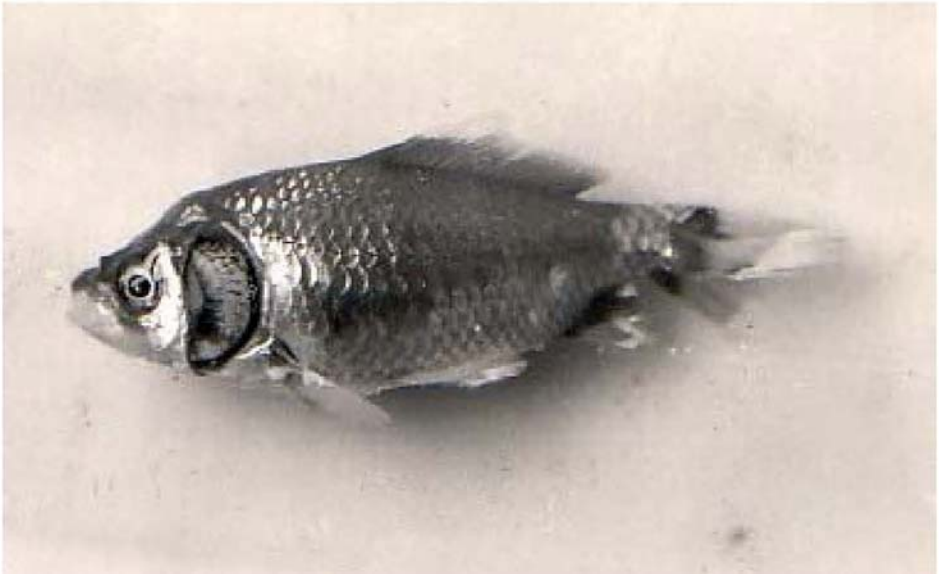

Figure 4.3. Goldfish infected with *F. columnare*. Lesion on the periphery of gills and fins gradually extending towards the body surface.

The influence of water quality on F. columnare infection has been studied by many authors. Fijan (1968) indicated that *F. columnare* could persist for long periods in water of high hardness and organic matter content. Survival and growth of *F. columnare* are affected by the ionic composition of water (Wakabayashi and Egusa 1972, Chowdhury and Wakabayashi 1988a,b). Columnaris disease did not occur in 3.0 or 9.0‰ salinity, and 31–39% fewer channel catfish (*Ictalurus punctatus*), goldfish (*Carassius auratus*) and striped bass (*Morone saxatilis*) died in 1.0‰salinity than in freshwater (Altinok and Grizzle 2001). Hanson and Grizzle (1985) indicated that nitrite at a concentration of 5 ppm enhanced *F. columnare* infection. Sugimoto et al. (1981) found that *F. columnare* grew very well on particles of fresh meal derived from the break-up of pelleted diets in water. In an experiment, transmission of columnaris disease to healthy fish was enhanced by adding a small quantity of feed pellets to an aquarium containing both diseased and healthy fish, but no transmission occurred without addition of the particulate feed matter (Sugimoto et al. 1981).

Bacterial culture and biochemical characterization has been employed for diagnosis of columnaris disease. However, this traditional culture technique requires several days to complete. Immunological techniques such as agglutination test, enzyme-linked immunoassay (ELISA) (Shoemaker et al. 2003) and indirect fluorescent antibody test (Panangala et al. 2006) have been used for detection of *F. columnare* infection. Several researchers have developed identification methods for *F. columnare* using PCR targeted 16S ribosomal DNA (Toyama et al. 1996, Bader et al. 2003). A PCR detection method based on the 16S-23S rDNA intergenic spacer region (IRS) of the ribosomal RNA operon was used for detection of *F. columnare* in channel catfish tissues and in tank water (Welker et al. 2005). Panangala et al. (2007) developed a multiplex PCR (m-PCR) for simultaneous detection of three bacterial fish pathogens, *F. columnare, Edwardsiella tarda,* and *Aeromonas hydrohila* in warm water aquaculture. In their study, they found that *A. hydrophila* outcompetes not only *F. columnare* but also *E. ictaluri.*

4.5. Control

Environmental control along with good rearing practice might provide a means of controlling columnaris disease. Auxiliary cold water, if available, is extremely beneficial even if it cools the water by only a few degrees (Wood 1979). Both salt (4 and 2%) and acidic baths failed to prevent fish mortality, but the mortality rate was lower in rainbow trout treated with 4% salt bath compared to a control group (Suomalainen et al. 2005b). The incidence of *F. columnare* in fish might even be reduced by adding significant numbers of competitive bacteria susceptible fish ponds before *F. columnare* became established on the fish body (Chowdhury and Wakabayashi 1989, Sumalainen et al. 2005c). Suomalainen et al. (2005a) showed that high rearing density, together with high temperature, were the main factors influencing mortality during a *F. columnare* outbreak. Shoemaker et al. (2003) demonstrated that in the absence of natural food juvenile channel catfish should be fed at least once every other day to apparent satiation to maintain normal physiological function and improve resistance to *F. columnaris*.

An excellent review on chemotherapeutics and compounds which had been commonly used for treating columnaris disease was provided by Amend (1970). Heavy metals such as copper sulfate ($CuSO_4$), potassium permanganate ($KMnO_4$), PMA (pyridylmercuricacetate) were used for many years, but their use as therapeutants is now restricted in most countries including UK, Japan and USA because they accumulate in the tissues of treated fish. An immersion flush exposure of NH_4Cl at 46.3mg/L (normally yielding 15mg/L total ammonia nitrogen) served to lower the channel catfish mortality caused by *F. columnare* (Farmer et al. 2011). Chloramine-T (*n*-chloro-*para*-toluene sulfonamide sodium salt) or Diqat (6,'-dihydrodipyrido 1,2-a: 2', 1'-c pyrazidinium dibromide) have been used extensively for treating columnaris disease in the USA (Altinok 2004, Thomas-Jinu and Goodwin 2004, Darwish and Mitchell 2009). For systemic infections, sulfonamide or antibiotics are added to the food. Administration of florfenicol at a dosage of 10 or 15mg/kg body weight for 10 days was efficacious for the control of mortality from *F. columnare* infection in channel catfish (Gaunt and Gao 2010, Darwish et al. 2012).

Studies on oral, parenteral and immersion vaccination of channel catfish against *F. columnare* were carried out but results were inconclusive (Schachte and Mora 1973). Moor et al. (1990) demonstrated the feasibility of immunizing channel catfish against columnaris disease by immersion vaccination with formalin-inactivated bacterins. A commercial vaccine against columnaris disease is available under the registered name Aquavac-Col (Intervet / Schering-Plough Animal Health) and the main active ingredient for the vaccine is a rifampin-resistant mutant of *F. columnare* (Olivares-Fuster and Arias 2011).

4.6. Recent Topics

Shoemaker et al. (2008) carried out immersion challenge experiments to ascribe virulence of genomovar I and II isolates to channel catfish. Their results demonstrated that genomovar II ($n = 4$) isolates were significantly more virulent to channel catfish fry (92-100% mortality) than genomovar I ($n = 3$) isolates. Klesius et al. (2008) reported that genomovar II isolates were more strongly chemotactic to channel catfish mucus than

genomovar I isolates. Olivares-Fuster et al. (2011) demonstrated that the cells of a genomovar II strain adhered to channel catfish gill in higher numbers within 1 h post-challenge. It is plausible that genomovar II strains could more efficiently adhere to the epithelial tissues and mucus coverings of catfishes. However, further research is needed to confirm if all genomovar II strains are indeed more effective at colonizing gills of channel catfish than genomovar I strain (Olivare-Fuster et al. 2011).

5. BACTERIAL COLD-WATER DISEASE

Hisatsugu Wakabayashi

5.1. Synopsis

Bacterial cold water disease (BCWD) caused by *Flavobacterium psychrophilum* is a serious disease in freshwater fish, particularly salmonid fish and ayu, worldwide. The epizootics are most prevalent at low temperature. The bacterial cells are Gram-negative, slender rods measuring 0.5×2-7 □m, exhibiting weak gliding motility. The clinical signs of BCWD depend on the age of affected fish species. In coho salmon fingerlings, the erosion of tissue in peduncle area is a classic characteristic early in the epizootics. Chemotherapy with antibiotics is still the most effective treatment method, but acquired resistance of *F. psychrophilum* is a major challenge. Currently, there are no vaccines commercially available to prevent BCWD.

5.2. Introduction

Bacterial cold-water disease (BCWD) is caused by *Flavobacterium psychrophilum* (formerly *Flexibacter psychrophilus* and *Cytophaga psychrophila*). Davis (1946) described it as ‘peduncle disease’ based on the characteristic pathology that was associated with the peduncle of the diseased rainbow trout (*Oncorhynchus mykiss*) in West Virginia, USA. Although Davis could not isolate the causative agent, he observed a number of long thin bacteria within the lesions of affected fish. The etiologic bacterium was originally isolated from diseased coho salmon (*Oncorhynchus kisutch*) in Washigton, USA, in 1948 by Borg (1960). He proposed the name *Cytophaga psychrophila* for this organism. The disease became known as ‘bacterial cold-water disease’ or ‘low-temperature disease’ because epizootics were most prevalent at low water temperature.

BCWD was believed to be limited to North America until its outbreaks occurred among rainbow trout in Germany (Weis 1987) and France (Bernardet et al. 1988). In Europe, the disease is called as ‘rainbow trout fry syndrome’ (RTFS) (Lorenzenn et al. 1997), ‘visceral myxobacteriosis’ (Baudin-Laurencin et al. 1989), or ‘fry mortality syndrome’ (FMS) (Lorenzenn et al. 1991). *F. psychrophilum* has been isolated in USA, Canada (Lumsden et al. 1996), Germany, France, UK (Santos 1992), Northern Ireland (Lorenzen et al. 1991), Denmark (Lorenzen et al. 1991), Spain (Toranzo and Barja 1993), Swizterland (Lorenzen and Olesen 1997), Finland (Wiklund et al. 1994), Norway (Lorenzen and Olesen 1997), Sweden (Madetoja et al. 2001), Estonia (Madetoja et al. 2001), Turkey (Kum et al. 2011) , Japan (Wakabayashi et al. 1991), Korea (Lee and Heo 1998), Australia (Schmidtke and Carson 1995), Chile (Bustos et al. 1995), Peru (Lindstrom et al. 2009). Although outbreaks commonly occur among salmonids, BCWD

also affects carp (*Cyprinus carpio*), crucian carp (*Carassium carassius*), eel (*Anguilla anguilla*), and tench *(Tinca tinca*) (Lehmann et al. 1991), ayu (*Plecoglossus altivelis*) (Wakabayashi et al. 1994), chub (*Zacco platypus*) (Iida and Mizokami 1996), gobies (*Chaenogobius urotaenia* and *Rhinogobius brumneus*), and dace (*Trybolodon hakonensis*) (Amita et al. 2000).

The importance of BCWD has led to a significant volume of publications, which have been adequately reviewed by various authors (Wood 1979, Holt et al. 1993, Nematollahi et al. 2003a, LaFrentz and Cain 2004, Cipriano and Holt 2005, Barnes and Brown 2011, Starliper 2011).

5.3. Disease Agent

Cytophaga agar (CA) (Anacker and Ordal 1959) is the most commonly used medium for isolation of *F. psychrophilum* from diseased fish. Colonies grown for 2-5 days at 15-20°C on CA are moist, yellow, circular, convex, smooth and non-adherent, 1-5 mm in diameter. Most strains produce colonies with a thin spreading irregular edge. Some strains produce colonies with a regular edge or a mixture of the two types (Figure 5.1).

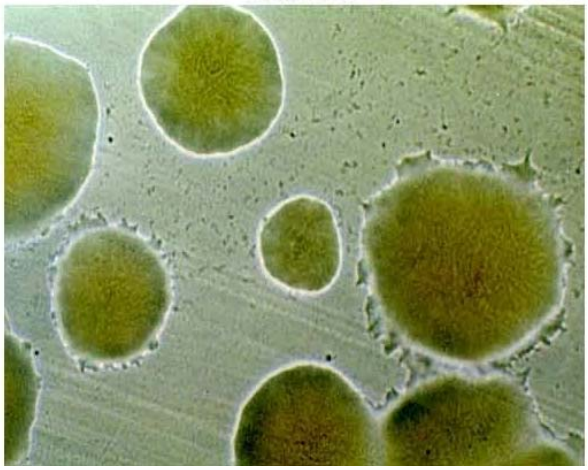

Figure 5.1. Colonies of a *F. prychrophilum* strain on Cytophaga agar, showing a mixture of regular and irregular periphery.

The cells are Gram-negative, slender rods measuring 0.5×2-7 μm (Figure 5.2). The bacteria exhibit gliding motility on wet mount, but gliding is slow and difficult to observe. Holt et al. (1993) described the physiological characteristics of 28 strains of *F. psychrophilum* isolated from various salmonids in USA as follows. All 28 strains grew in supplemented TYE broth at 5-23°C, 18 strains grew slowly at 25°C, and no growth occurred at 30°C. All 28 strains grew in the presence of 0.5 and 1.0% NaCl but none grew in 2.0%. Catalase is positive, and cytochrome oxidase is negative. Flexirubin pigment is present in cells. Ammonium is produced, and hydrogen sulfide, indole, acetylmethl carbinol are not produced. Nitrate is not reduced. Casein, gelatin, albumin, and collagen are degraded. Agar, cellulose, carboxymethyl cellulose, starch, and chitin are not degraded. Degradation of elastin and tyrosine are variable. No acid is produced from simple or complex carbohydrates. Bernardet and Kerouault (1989) reported the presence of cytochrome oxidase but the reaction was weak. The DNA base composition (G+C

content) were reported 33.2-35.3 mol% with a mean of 34.3% for 13 strains (Holt et al. 1993), and 32.5-33.8mol% with a mean of 33.4% for 3 strains (Bernardet and Kerouault 1989). Duchaud et al. (2007) reported the complete genome sequence of the virulent strain JIP02/86 (ATCC49511) of *F. psychrophilum* that contained 32.54% G+C content.

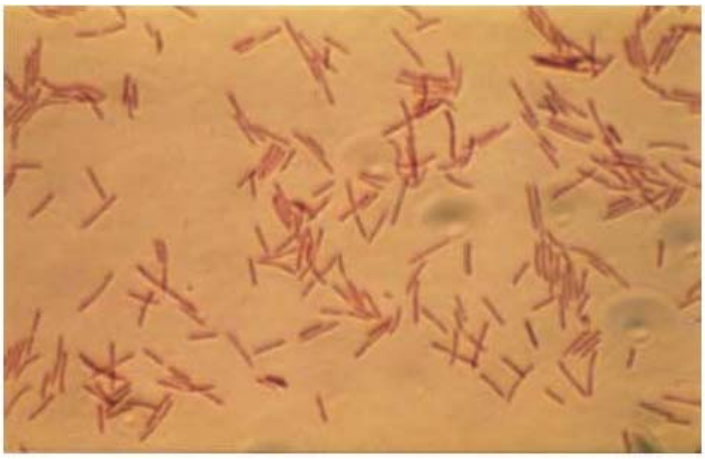

Figure 5.2. Gram-stained *F. prychrophilum* cells, measuring 0.5 ×2 – 7 μm.

Strains of *F. psychrophilum* from salmonids in USA were reported to share common antigen(s) by various authors (Pacha 1968, Pacha and Porter 1968, Bullock 1972, Holt et al. 1993). On the basis of the absorption analysis with thermo-stable antigens, Wakabayashi et al. (1994) and Izumi and Wakabayashi (1999) demonstrated the existence of antigenic diversity within the species, and established three O groups (O1, O2 and O3). The typing system developed by Lorenzen and Olesen (1997) recognized the Tp^T, Th (subtype Th-1 and Th-2) and Fd serotypes. Mata et al. (2002) found seven host-dependent serovars from 34 isolates worldwide. Serovar 1, previously described as O1 or $Fp^{T,}$ was only found in strains isolated from salmon. Serovars 2 and 3, previously described as O3 or Th and Fd, were only found in rainbow trout. Serovars 4, 5, 6 were found in isolates from eel, carp, and tench, respectively. Serovar 7 was equivalent to serotype O2 previously only found in strains from ayu in Japan. Using *Cla*I, *Hae*III and *Pvu*II restriction enzymes in ribotyping analyses 13 different genotypes were demonstrated and a possible relationship between serotype Fd and genotype F1 was determined (Madetoja et al. 2001). Izumi et al. (2003) reported that *F. psychrophilum* could be divided into two genotypes, A and B, by the polymorphism in an anonymous product of 290 bp that was amplified with universal primers for gyrase subunit B gene. Genotype A was found only in isolates from ayu, (*n*=109), while genotype B was found in isolates from coho salmon (*n*=11), ayu (*n*=35), rainbow trout (*n*=43) and other fishes (*n*=44). Yoshiura et al. (2006) identified the 290 bp fragment as a part of coding region of peptidyl-polyl cis-trans isomerase C (*ppiC*) gene. A 326 bp DNA fragment that differentiated genotypes A and B was amplified with a new PCR primers designed for *ppiC* gene (Yoshiura et al. 2006). The evidence that ayu has its own peculiar type of *F. psychrophilum* provoke a question where the type is originated. A hypothesis is that selection for the adapted mutants of *F. psychrophilum* might have occurred in Lake Biwa, the largest lake in Japan, because the single outlet of the lake, Seta River, is dammed and all the population of ayu are landlocked in the lake (Wakabayashi 2009). Nicolas et al. (2008) examined the nucleotide polymorphisms at 11 protein-coding loci of the core genome in a set of 50 strains from 10

different host fish species and four continents. The analysis provided no clues that the initial range of the bacterium was originally limited to North America, and suggested that human activities might enable the main two clonal complexes (CC1 and CC2) spread worldwide (Nicolas et al. 2008).

Strains of *F. psychrophilum* differ widely in virulence (Holt et al. 1993, Madsen and Dalsgaard 2000). Dalsgaard (1993) reviewed various reports concerning the factors determining virulence of *F. psychrophilum.* Nematollahi et al. (2003b) made a comparison between the adhesion capacity of a high and low virulence *F. psychrophilum* strain by using a gill perfusion model and demonstrated that the high virulent strain attached more ready to the gill tissue than did the low virulence. Furthermore, the adhesion of the high virulent strain to the gill tissue was influenced by environmental factors such as organic material, nitrite and temperature. Analysis of 29 isolates of *F. psychrophilum* indicated that the isolates formed four groups based on the presence or absence of certain proteases visualized by substrate SDS-PAGE. *In vivo* infectivity experiments with juvenile steelhead and coho salmo indicated some association between protease group and virulence (Bertolini et al. 1994). Ostland et al. (2000) shown that a crude extracellular preparation from a strain of *F. psychrophilum* had proteolytic activity in that it could degrade gelatin and type II collagen *in vitro* and can produce severe muscle necrosis in experimentally injected rainbow trout after 24 h at 8°C. Secardes et al. (2001) purified an extracellular protease, designated Fpp1 (*F. psychrophilum protease* 1), that cleaved gelatin, laminin, fibronectin, fibrinogen, collagen type IV, and to a lesser extent, collagen types I and II. Production of Fpp1 depended on factors such as calcium concentration, growth phase of the culture, and temperature. Nematollahi et al. (2005) reported that high virulence in *F. psychrophilum* appeared to be correlated with higher macrophage cytotoxicity and resistance to reactive oxygen species (ROC) and, therefore, with enhanced resistance to bacterial killing by rainbow trout macrophages. Nagai and Nakai (2011) demonstrated that the *in vitro* growth of *F. psychrophilum* isolates in host fish serum correlated well with their pathogenicity to host fish, particularly in ayu. All isolates (n=19) from ayu grew well with a 9- to 116-fold increase of colony forming unit (CFU) in ayu serum, while CFU decreased markedly in amago salmon (*Oncorhynchus masou ishikawae*) serum. Experimental infection by intraperitoneal injection showed that ayu isolates examined were all pathogenic to ayu but not to amago salmon.

5.4. Diagnostic Methods

Epizootics of BCWD commonly occur when water temperatures range between 4 and 10°C, but mortality generally abates as temperature approach 15-18°C (Cipriano and Holt 2005). In feral ayu, the disease occurs mostly at water temperature between 12 and 20°C, and a sudden drop in water temperature after heavy raining may have a major impact on the outbreaks (Wakabayashi 2009).

The clinical signs of BCWD differ with the age of affected fish. In coho salmon alevins, the skin covering the yolk sac becomes eroded and the sac may rupture. In fingerlings, the erosion of tissue in peduncle area is observed early in epizootics of BCWD, and later in the outbreak these lesions are found at various locations such as anterior to the dorsal fin, on the lateral side, ventrally, near the vent or on the lower (Cipriano and Holt 2005). In some outbreaks, moribund coho salmon with no external skin lesions, display dark

pigmentation on one side of the body, exhibit dorsal swelling just posterior to the skull, and swim in spiral motions when agitated (Kent et al. 1989). Juvenile coho salmon with aberrant spinal columns occurred several months after symptoms of BCWD disappeared, and the incidence is always greatest at hatcheries where BCWD was most severe (Conrad and DeCew 1967). Such fish often have to be discarded and can result in significant economic loss.

In case of 'rainbow trout fry syndrome' (RTFS), fish weighing 0.2-1 g are the most frequently affected. They exhibit dark coloration of the skin, ascites and exophthalmia. The fry suffered from a severe anaemia causing extremely pale gills. The most consistent internal lesion is spleen hypertrophy often associated with liver discoloration (Berma; det et al. 1988, Lorenzen et al. 1991). *F. psycrophilum* infection at the fry stage may result in an increased occurrence of vertebral column deformities in farmed rainbow trout (Madsen and Dalsgaard, 1999, Madsen et al. 2001).

The clinical signs of ayu infected with *F. psychrophilum* are similar to those of salmonid species. Skin and muscle peduncle lesions are observed first in juvenile ayu (Figure 5.3). In epizootics of BCWD among feral adult ayu, deep dermal ulcerations with necrosis of underlying musculature are found at various locations on the lateral side (Figure 5.4). Most of the affected feral ayu show pale gills, liver discoloration, and spleen hypertrophy (Iida and Mizokami, 1996).

Figure 5.3. Peduncle lesions caused by *F. psychrophilum* in juvenile ayu.

Figure 5.4. Deep dermal ulceration with necrosis of the underlying musculature caused by *F. psychrophilum* in feral adult ayu

For presumptive diagnosis, a microscopic examination of an imprint of spleen tissue that have been air dried and stained with safranin for one minute often will reveal many cells with typical *F. psychrophilum* morphology (Cipriano and Holt 2005). For definitive diagnosis, bacteria should be isolated on an appropriate medium and identified as *F. psychrophilum.* Cytophaga agar (Anacker and Ordal 1959) is the most commonly used, but there have been several reports of improved culture media for *F. psychrophilum* (Holt et al. 1993, Lorenzen 1993, Daskalov et al. 1999, Michel et al. 1999, Cepeda et al. 2004, Alvarez and Gijarro 2007). Other sensitive diagnostic techniques than bacterial culture have been employed to detect *F. psychrophilum* in fish and its surroundings. These include serological methods such as immuno-fluorescence method (Lorenzen and Karas 1992, Izumi and Wakabayashi 1997, Amita et al. 2000, Vatsos et al. 2002, Lindstrom et al. 2009, Long et al. 2012), immuno-enzyme method (Evensen and Lorenzen 1996, 1997, Aikawa 1998) and enzyme-linked immune-sorbent assay (ELSA) (Rangdale and Way 1995, Mata and Santos 2001, Lindstrom et al. 2009, Long et al. 2012). Molecular techniques have also been employed for non-culture based detection of *F. psychrophilum,* including restriction fragment length polymorphism (Nilson and Strom 2002), *in situ* hybridization (Liu et al. 2001), and polymerase chain reaction (PCR) (Toyama et al. 1994, Bader and Shotts 1998, Urdaci, et al. 1998, Cepeda and Santos 2000). Nested PCR assays have been adopted to detect low levels of *F. psychrophilum* from fish tissues and particularly from its surroundings (Izumi and Wakabayashi 1997, Wiklund et al. 2000, Baliarda et al. 2002, Taylor and Winton 2002, Izumi et al. 2005, Crumlish et al. 2007). Suzuki et al. (2008) compared the sensitivity and specificity of PCR methods targeting 16S rDNA, DNA gyrase subunit genes (*gyr A*, *gyrB*) and *ppiC* for detection of *F. psychrophilum*, and concluded the PCRs targeting gyrB and ppiC seem to be preferable because of no false-positives. Del Cerro et al. (2002a) and Altinok (2011) developed maltiplex PCR methods for the simultaneous detection of three and four major fish pathogens including *F. psychrophilum*, respectively. Del Cerro et al. (2002b) also developed a new detection method for *F. psychrophilum* based on a TaqMan PCR assay. Orieux et al. (2011) described quantification of F. psychrophilum in rainbow trout tissues by qPCR.

5.5. Control

Chemotherapy with antibiotics is still an important method of BCWD control. Oxytetracycline (OTC), amoxicillin (AMS), oxolinic acid (OXA) and florfenicol (FLO) have been widely used around the world (Bruun, 2000, Lumsden et al. 2006). In USA, OTC and FLO are approved for treatment of BCWD in captive-reared fish (Starliper 2011). In Japan, the approved drugs are OTC, OXA, FLO, and sulfuisozole for freshwater-cultured rainbow trout, and OXA, FLO, and sulfuisozole for ayu. However, it has been reported that acquired resistance of *F. psychrophilum strains* exists to various antibiotics (Rangdale et al. 1997, Soule et al. 2005, del Cerro et al. 2010, Kum et al. 2008, Henriquez-Nunez et al. 2012).

Although no licensed vaccines are currently available for prevention of BCWD, several attempts to vaccinate fish against *F. psychrophilum* have been published. Various levels of protection were demonstrated in immunization trials with whole-cell bacterins administered by immersion and/or injection routes (Holt 1993, Obach and Laurencin 1991, Rahman et al. 2000, LaFrentz et al. 2002, Madetoja et al. 2006). Kondo et al.

(2003) demonstrated the effectiveness of oral vaccination against BCWD in ayu. Recent studies have aimed at the development of subcellular vaccines. Rahman et al. (2002) reported that the outer-membrane fraction of *F. psychrophilum* induced significantly higher protection against BCWD in both rainbow trout and ayu compared to the whole-cell bacterin. Plant et al. (2009) demonstrated high antibody resposes in rainbow trout to heat shock proteins 60 and 70. LaFrentz et al. (2011) identified 15 proteins of *F. psychrophilum* by immunoproteomics and suggested that antibodies apecific for outer membrane protein OmpA, trigger factor, ClpB, elongation factor G, gliding motility protein GldN and a conserved hypothetical protein may be important for protective immunity from BCWD. A few studies have dealt with live attenuated vaccine against *F. psychrophilum* infection. LaFrenz et al. (2008) demonstrated that the immersion delivery of the rifampicin resistant 259-93B.17 strain stimulated protective immune responses in fish at 10 weeks post-immunization. Gliniewicz et al. (2012) described that the 259-93B.17 strain havoured a mutation in the *rpoB* gene consistent with resistance to rifampicin. Alvarez et al. (2008) reported that a mutant in one of two *exbD* loci of a TonB system in *F. psychrophilum* showed attenuated virulence and conferred protection against BCWD.

Because *F. psychrophilum* has been detected in fluid surrounding the eggs in sexually mature salmonids, iodophore treatment of eggs is routinely practiced to reduce microbial contamination of the egg surface (LaFrentz and Cain 2004, Cipriano and Holt 2005). However, *F. psychrophilum* presents within egg contents, not just in the surrounding fluids or on the egg surface (Brown et al. 1997, Kumagai et al. 2000, Taylor 2004, Cypriano 2005). Broodstock or egg culling and segregation programs can reduce the probability of BCWD epizootics in progeny at select aquaculture facilities, and the ELIZA is an appropriate tool to screen broodstock and provides an indication of infection severity (Lindstrom et al. 2009, Long et al. 2012).

5.6. Recent Topics

Recent researches suggest that selective breeding for innate resistance may offer a promising tool to control BCWD. Nagai et al. (2004) showed that amphidromous stock of ayu was significantly lower in susceptibility to *F. psychrophilum* challenges than domesticated and land-locked stocks. Henryon et al. (2005) demonstrated additive genetic variation for resistance to *F. psychrophilum* in a Danish rainbow trout population. These studies indicated a favorable potential for selective breeding for increased resistance. Haddi et al. (2008) characterized the phenotype of *F. psychrophilum* resistance and susceptible families of fish as they increased in size > 300-fold, and they showed a positive correlation between disease resistance and normalized spleen weight. Silverstein et al. (2009) demonstrated that rainbow trout survival after *F. psychrophilum* injection challenge was a moderately heritable trait in their broodstock population, indicating favorable implictions for selective breeding for increased disease resistance. More recently, a paper entitled as ‘Selective breeding of food sized rainbow trout against Flavobacteriosis’ was presented in the 3rd International Conference on the Members of the Genus *Flavobacterium* (LaPatra et al. 2012).

6. RED SPOT DISEASE

Toshihiro Nakai

6.1. Synopsis

Red spot disease was first reported in 1972 from farmed Japanese eel (*Anguilla japonica*) in Japan and then from farmed European eel (*A. anguilla*) in European countries, with its characteristic subepidermal petechiae on the body surface and severe mortalities. Thereafter, the disease was recorded in various non-anguillid farmed species which were reared mostly under blackish or saltwater conditions. The causative agent, *Pseudomonas anguilliseptica*, is relatively psychrophilic and halophilic, and homogeneous in the phenotypic and genetic characteristics. *P. anguilliseptica* is thought to be a typical facultative pathogen because of its low virulence in experimental infection.

6.2. Introduction

Red spot disease, or 'sekiten-byo' in Japanese, was first described in Japanese eel (*Anguilla japonica*) at Japanese commercial farms in 1972, and the disease had caused serious economical damages in many eel farms for successive several years in Japan (Wakabayashi and Egusa, 1972; Muroga, 1978). Typical clinical sign of the disease was subepidermal petechiae on the body surface, and the name 'red spot disease' was due to this conspicuous external sign (Figure 1). A histopathological examination revealed that manifestation of petechial hemorrhages in the body appeared in an advanced stage of the disease (Miyazaki and Egusa, 1977). The disease in Japanese eel was also confirmed in Taiwan in 1978 (Kuo and Kou, 1978) and then recorded in cultured European eel (*A. anguilla*) in Japan, Scotland, Denmark, France and the Netherlands (Jo et al, 1975; Stewart et al, 1983; Mellergaard and Dalsgaard, 1987; Michel et al, 1992; Haenen and Davidse, 2001). Compared with Japanese eel, European eel was relatively less susceptible to the disease (Jo et al, 1975; Haenen and Davidse, 2001).

Thereafter, the disease was recorded in various non-anguillid cultured fish species which were reared mostly under blackish or saltwater conditions in France, Spain, UK, Denmark, the Netherlands, Finland, Canada and Japan. These include black sea bream (*Acanthopagrus schlegeli*) (Nakajima et al, 1983), striped jack (*Pseudocaranx dentex*) (Kusuda et al, 1995), Atlantic cod (*Gadus morhua*) (Ferguson et al, 2004 ; Balboa et al, 2007), orange-spotted grouper (*E. coioides*) (Al-Marzouk, 1999), gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*) (Berthe et al, 1995; Domenech et al, 1997), black spot seabream (*Pagellus bogaraveo*) (Lopez-Romalde et al, 2003), salmonids such as Atlantic salmon (*Salmo salar*), sea trout (*S. trutta*), rainbow trout (*Oncorhynchus mykiss*), whitefish (*Coregonus* sp.) (Wiklund and Bylund, 1990; Wiklund and Lonnstrom, 1994) and ayu (*Plecoglossus altivelis*) (Nakai et al, 1985a). In the case of ayu, which is commonly cultured in freshwater ponds, infection might have been established in estuary where fish were caught as seeds for culture. The causative bacterium was also isolated from wild Baltic herring (*Clupea harengus membras*) with eye lesions (Lonnstrom et al, 1994).

Figure 6.1. *Pseudomonas anguilliseptica* infection (red spot disease) of Japanese eel showing intensive petechial hemorrhages on the skin

Epizootics of the disease in farmed Japanese eel in Japan prevailed mainly in early spring and sporadically in autumn when water temperature of the ponds ranged 10˚C to 20˚C, and ceased at 27˚C in early summer. Another epizootiological factor of the disease in Japanese eel farms was that farm ponds were located near the seashore and thus underground water used for fish rearing contained salinity (Cl 0.27-6.29 ppt) (Muroga et al, 1973). In cases of European eel, the disease was recorded at 23-25˚C and 16˚C in the Netherland and Denmark, respectively (Mellergaard and Dalsgaard, 1987; Haenen and Davidse, 2001). In marine fishes, the disease occurred during winter months when water temperature was below 16˚C in France and Spain (Berthe et al, 1995; Tranzo et al, 2005) or between 15˚C and 18˚C in salmonids in Finish coasts (Wiklund and Bylund, 1990). The most common clinical sign of the disease is haemorrhagic petechia on the skin. Petechial hemorrhages were also noticeable in the peritoneum and the adipose tissue of visceral organs in affected salmonids (Wiklund and Lonnstrom, 1994). The disease in Atlantic cod, Baltic herring and gilthead sea bream was often associated with eye lesions (Lonnstrom et al, 1994; Berthe et al, 1995 ; Ferguson et al, 2004).

6.3. Disease Agent

The causative agent of red spot disease, *Pseudomonas anguilliseptica*, is a Gram-negative, aerobic and motile rod, producing no acid from glucose and other carbohydrates. Growth of the bacterium on conventional agar media is rather slow and colonies are entire, convex, translucent and viscid. *P. anguilliseptica* is rather uniform in the biochemical characteristics regardless of the source of isolation, with a few exceptions. The bacterium grows in nutrient broth with NaCl 0-4% (optimum 0.5-1%) and at temperatures from 5˚C to 30˚C (optimum 15-25˚C). The cells are motile with a single polar flagellum but lose motility when cultured at 25˚C or over. Interestingly, the bacterium could survive in seawater or diluted seawater (Cl higher than 1.9 ppt) for more than 200 days, while it perished in freshwater within a day (Wakabayashi and Egusa, 1972; Muroga et al, 1977). Addition of seawater or Mg^{++} in culture media enhances motility of the cells in a wet mount method.

Electron microscopy revealed a capsule-like envelope on cell surface of *P. anguilliseptica* (Wakabayashi and Egusa, 1972). A series of serological analysis on Japanese, Taiwanese,

and Scotish isolates from eels demonstrated that the bacterium had a common heat-stable antigen (O antigen). However, based on a heat-labile antigen (stable at 100˚C for 30 min but labile at 100˚C for 120 min or 121˚C for 30 min), designated as K antigen, it was divided into two serotypes; K^+ type (K antigen-possessing) and K^- type (K antigen-lacking) (Nakai et al, 1981, 1982). These serotypes correlated well with their experimental virulence to eels; K^+ type was virulent to both Japanese and European eels but K^- type was avirulent to both species (Nakai and Muroga, 1982; Nakai et al, 1985b). *P. anguilliseptica* isolates from ayu also had K antigen (K^+-2), which was differentiated from that (K^+-1) of the eel isolate (Nakai et al, 1985a). Furthermore, it was shown that K antigen-related resistance to serum (complement)-killing of fish correlated well with the virulence of the isolates (Nakai, 1985) (Table 6.1). The Finnish isolates from salmonids were similar to serotype of the ayu isolates (Wiklund and Bylund, 1990). On the other hand, two different O serotypes were described for non-eel isolates and eel isolates; serotype O1 for isolates from turbot, sea bream, sea bass, herring and salmonids, and serotype O2 for isolates from Japanese and European eels (Lopez-Romalde et al, 2003; Balboa et al, 2007). This serotyping correlated with genotyping by randomly amplified polymorphic DNA (RAPD) analysis (Lopez-Romalde et al, 2003).

Fish or serum source	**Virulence to fish**			**Resistance to serum-killing**		
	K^+-1*[1]	K^+-2	K^-	K^+-1	K^+-2	K^-
Japanese eel	++*[2]	+	-	++	+	-
Bluegill	++	nd*[5]	-	++	nd	-
European eel	+*[3]	nd	-	+	nd	-
Ayu	+	++	nd	+	++	nd
Carp	-*[4]	-	-	-	-	-
Goldfish	-	nd	-	-	nd	-
Tilapia	-	-	-	-	-	-
Rainbow trout	-	nd	-	-	nd	-

*[1] serotype
*[2] high virulence (LD50: 10^6 cfu/fish) or high serum-resistance
*[3] low virulence (LD50: 10^{8-9} cfu/fish) or high serum-resistance
*[4] no virulence or no serum-resistance
*[5] no data

Table 6.1. Comparison of virulence to fish and resistance to serum-killing among *Pseudomonas anguilliseptica* serotypes (from (Nakai et al, 1985a; Nakai, 1985))

Several fish species were tested for their susceptibility to a virulent strain (K^+-1 type) of *P. anguilliseptica* by intramuscular injection (Muroga et al, 1975; Uno, 1976). Japanese eel was more susceptible to the pathogen than European eel. Ayu, bluegill (*Lepomis macrochirus*) and loach (*Misgurnus anguillicaudatus*) were highly susceptible, and carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) were slightly susceptible to the pathogen, while rainbow trout, amago (*Oncorhynchus rhodrus* f. *macrostoma*), kokanee salmon (*O. nerka* f. *adonis*) and iwana (*Salvelinus pluvius*) were not susceptible. A similar experimental infection with a K^+-1 type strain showed that the LD50 to Japanese eel was about 10^6 cfu/fish (Nakai et al, 1985). In a dip method, which fish were kept at

tanks containing 10^{6-7} cfu/ml of the bacterium, infection was established if diluted seawater was used as rearing water, while gastral administrations of the bacterium failed to cause mortality (Muroga and Nakajima, 1981). When Japanese eels were challenged by intramuscular injection with the K^+-1 type strain under different water temperatures, fish died at 12˚C or 20˚C with high cell numbers (10^{8-10} cfu/g or ml) in the blood and organs, but not at 28˚C (Nakai et al, 1985). The LD50 of the turbot isolate to juvenile turbot by intraperitoneal injection was 10^6 cfu/fish (Magi et al, 2009).

6.3.1. Diagnostic methods

P. anguilliseptica is easily isolated with abundant colonies on conventional nutrient agar media from various organs of diseased fish. The bacterium is biochemically homogeneous and can be differentiated from the other fish-pathogenic pseudomonads (*P. fluorescens*, *P. putida*, *P. chlororaphis* and *P. plecoglossicida*) by negative reactions in carbohydrate utilization. Serological and RAPD techniques are available for serotyping and genotyping of *P. anguilliseptica* as well as rapid diagnosis of the disease (Horiuchi and Kohga, 1979; Nakai et al, 1981; Lopez-Romalde et al, 2003). PCR-based techniques have been developed for rapid identification of *P. anguilliseptica* or sensitive detection of the pathogen from fish (Blanco et al, 2002; Romalde et al, 2004; Beaz-Hidalgo et al, 2008).

6.4. Control

P. anguilliseptica was sensitive to some antibiotics (Wakabayashi and Egusa, 1972; Wiklund and Bylund, 1990), and treatments with oxolinic acid and nalidixic acid were effective to experimentally infected Japanese eels (Jo, 1978). However, chemotherapy is not so effective in eel farms mainly due to the fact that the disease occurs in early spring when fish have poor appetite at lower water temperature. Treatment of Atlantic salmon with oxytetracycline had only a limited effect (Wiklund and Bylund, 1990).

As mentioned previously, the disease occurs preferably in Japanese eels farmed in brackish water ponds in spring and autumn. The epizootiological features were supported by the experimental results of physiological and pathological characteristics of the pathogen. Based on these findings, some control measures were proposed (Muroga, 1978). In the areas where the epizootic has been prevailing, eels should be cultured in freshwater ponds and/or the water temperature should be kept at 26˚C or higher. Particularly, the temperature manipulation was so efficacious that the epizootic had burnt low at late 1970s and completely disappeared since 1980s in eel farms in Japan. Development of green-house culture system for Japanese eel, where water temperature is constantly kept at about 26˚C for optimum growth of eel, greatly contributed to eradication of the disease.

The temperature manipulation, however, is not applicable for salmonids and other coldwater fish species or cage-cultured marine fishes in the open sea. It was confirmed under laboratory setting that both antibody response and protection in Japanese eels immunized by injection with *P. anguilliseptica* bacterin (formalin-killed cells) incorporated with FCA were maintained over five months (Nakai and Muroga, 1979). A field vaccination trial was conducted in a commercial eel farm having history of red spot

disease. Japanese eels were injected intramuscularly twice with heat-killed (100˚C for 30 min) bacterin on the beginning of November in 1980. Red spot disease occurred in the pond from May to June in 1981 and the injection vaccination procedure proved to be effective against natural infection of *P. anguilliseotica* (Nakai et al, 1982). However, any successful results have not been obtained by either immersion or oral vaccination for eels. On the other hand, it was shown that non-mineral oil-adjuvanted bacterins were effective against experimentally induced disease in gilthead seabream and turbot (Tranzo et al, 2005).

The aforementioned epizootiological and pathological findings suggest that *P. anguilliseptica* is ubiquitous in salt or brakish waters, and wild fishes might serve as an important infection source, either vector or carrier, for farmed fish (Lonnstrom et al, 1994). Since *P. anguilliseptica* is possibly a typical facultative pathogen to any fish species, it is essential to reduce predisposing factors for controlling the disease (Mushiake et al, 1984).

7. EDWARDSIELLOSIS (EDWARDSIELLA ICTALURI)

Tomokazu Takano

7.1. Synopsis

Edwardsiella is a distinct taxon within the family Enterobacteriaceae, and includes three species, *Edwardsiella ictaluri* (Hawke, et al, 1981), *E. tarda* (Ewing et al, 1965) and *E. hosinae* (Grimont et al, 1980). *Edwardsiella hosinae* strains were mainly isolated from birds and reptiles (Grimont et al, 1980). Both *E. ictaluri* and *E. tarda* cause diseases in fish. More specifically, *E. ictaluri* is associated with freshwater fish species including ictalurid fish, whilst *E. tarda* has a broader host range amongst freshwater and marine fish species (Abbott and Janda, 2006; Evans et al, 2011). In this section information on *E. ictaluri* is discussed.

7.2. Introduction

Hawke (Hawke 1979) first reported undefined species of *Edwardsiella* from channel catfish (*Ictalurus punctatus*) suffering from enteric septicaemia. His later research (Hawke, et al, 1981) revealed that *Edwardsiella ictaluri* was the causative agent of enteric septicaemia of catfish (ESC), which is one of the most important infectious diseases of the catfish industry in the USA. The economic losses caused by ESC have been estimated to be US$20-60 million/year (Evans et al, 2011; Plumb and Vinitnantharat, 1993; Shoemaker et al, 2003). Besides catfish production in USA, the freshwater catfish (*Pangasius hypophthalmus*) industry in Southeast Asian countries also suffers from *E. ictaluri* infections (Ferguson et al, 2001; Crumlish et al, 2002 ; Yuasa et al, 2003).

7.3. Disease Agent

7.3.1 Characteristics

The type strain of *E. ictaluri* is ATCC 33202. It is a Gram-negative, rod-shaped bacterium which measures 0.5 by 1.25 μm after 18 to 48 h of culture on solid media. At 25°C it is

motile using peritrichous flagella. The optimum growth temperature and pH is between 25–30°C (Hawke, et al, 1981) and 7.0–7.5 (Plumb and Vinitnantharat, 1989), respectively. Growth occurs in 1.5% sodium chloride, but not 2% sodium chloride (w/v) (Waltman et al, 1986). *Edwardsiella ictaruli* produce catalase, lysine and ornithine decarboxylase, but not cytochrome oxidase and β-galactosidase (Waltman et al, 1986). It also ferments and oxidizes glucose while producing gas at 25–30°C, but not at 37°C (Hawke, et al, 1981; Waltman et al, 1986). *Edwardsiella ictaluri* does not produce indole and hydrogen sulfide, whilst *E. tarda* does (Hawke, et al, 1981). *Edwardsiella ictaluri* and *E. tarda* may be differentiated by these biochemical characteristics (Figure 7.1).

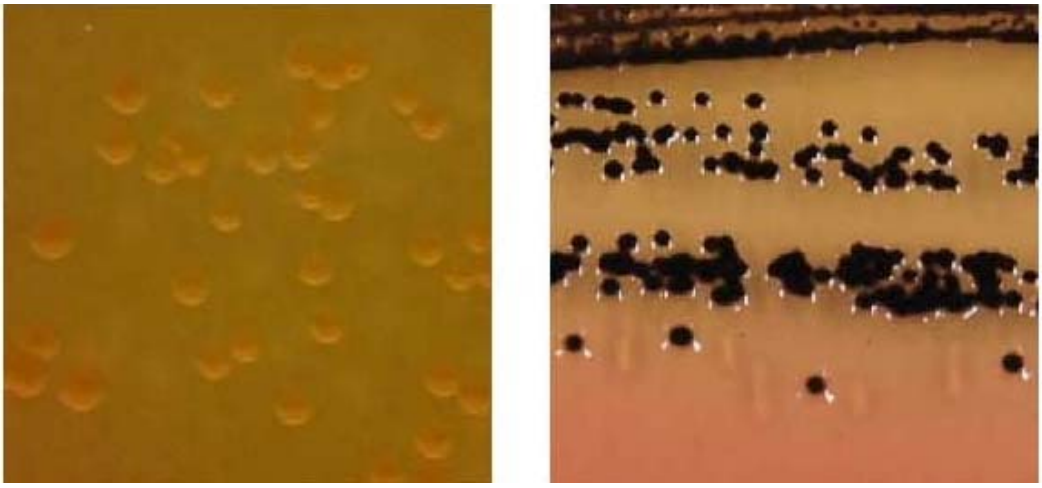

Figure 7.1. Colonies of *E. ictaluri* and *E. tarda* on SS agar. *Salmonella-Shigella* agar permits detection of hydrogen sulfide (H_2S) by the production of colonies with black centers. *Edwardsiella ictaluri* isolated from ayu does not produce H_2S (A), whilst *E. tarda* isolated from Japanese flounder produces H_2S (B).

7.3.2. Genome Size

The whole genome sequence of *E. ictaluri* 93-146, a wild-type isolate from a natural outbreak in Louisiana in 1993, has been determined. The completed genome of *E. ictaluri* 93-146 is 3,812,315 bp in length. A total of 3,783 protein-coding genes were predicted from the genome sequence. Of these, 2,007 genes have functional predictions. The sequence has an average G + C content of 57.4% [13].

7.3.3. Serological Classification

Plumb and Klesius (Plumb and Klesius, 1988), using *E. ictaluri* monoclonal antibody preparations, showed that 17 USA isolates were serologically identical. Furthermore, identical agglutination titers of *E. ictaluri*-specific rabbit antisera to isolates from different hosts and different geographic localities were reported (Plumb and Vinitnantharat, 1989). Bertolini et al. (1990) reported the strong cross reactivity of antisera prepared from catfish and non-ictalurid (green knife fish, *Eigemmannia virescens*) isolates. Therefore, it is believed that there is little serological diversity amongst *E. ictaluri* strains.

7.3.4. Molecular Classification

For intraspecific classification of *E. ictaluri*, molecular techniques allowed better resolution than serological techniques. Lobb et al. (1993) utilized western blot analysis of the proteinase K-treated cell lysates and Southern blot analysis of the cryptic plasmid DNAs, and they were able to differentiate *E. ictaluri* isolates. Amongst twenty (20) *E. ictaluri* isolates, four subgroups were identified using enterobacterial repetitive intergenic consensus (ERIC) PCR (Bader et al, 1998). Fingerprinting by amplified fragment length polymorphisms (AFLP) demonstrated that the madtom (*Noturus gyrinus*) isolates were genetically different from the other *E. ictaluri* isolates (Klesius et al, 2003). Recently, mass mortality of wild ayu (*Plecoglossus altivelis*) caused by *E. ictaluri* infection was reported in Japan. Isolates of *E. ictaluri* from ayu and isolates from *P. hypophthalmus* in Indonesia were classified into the same subgroups, whereas isolates from channel catfish in the USA were classified into another subgroup based on AFLP fingerprinting (Sakai et al, 2009).

7.3.5. Pathogenesis

Four families of catfish, Ictaluridae, Clariidae, Siluridae and Pangasiidae, are associated with *E. ictaluri* infection (Evans et al, 2011). In addition, Bengal danio (*Danio devario*), Chinook salmon (*Oncorhynchus tshawytscha*), European sea bass (*Dicentrarchus labrax*), green knife fish, Japanese eel (*Anguilla japonica*), rosy barb (*Puntius conchonus*), rainbow trout (*O. mykiss*), rudd (*Scardinius erythrophthalmus*), striped bass (*Morone saxatilis*), and white perch (*M. americana*) have been infected with *E. ictaluri* (Evans et al, 2011). Channel catfish is highly susceptible to *E. ictaluri*. An injection of 1.5×10^3 cells of the bacterium into the catfish was sufficient to cause 100% mortality (Plumb and Sanchez, 1983). From the data of an experimental challenge test amongst the non-catfish species, only tilapia (*Oreochromis aureus*) showed slight susceptibility, whilst Golden shiner (*Notemigonus crysoleucas*), bighead carp (*Aristichthys nobilis*) and largemouth bass (*Micropterus salmoides*) were completely resistant (Plumb and Sanchez, 1983). It is reported that the ayu has a relatively higher susceptibility ($LD_{50} = 1.3 \times 10^4$ CFU/fish) to *E. ictaluri* infection (Sakai et al, 2008).

Morrison and Plumb (1994) experimentally demonstrated the attachment of *E. ictaluri* on the olfactory mucosal surface of channel catfish. With regard to this attachment mechanism, it was reported that *E. ictaluri* possesses bacterial lectins to attach to specific sugar residues, including D-mannose, *N*-acetylneuraminic acid and L-fucose, of nasal mucosa (Wolfe et al, 1998). Thus, the olfactory organ of catfish appears to be important in the waterborne infection of *E. ictaluri* (Morrison and Plumb, 1994; Miyazaki and Plumb, 1985; Shotts et al, 1986). Intestinal mucosa is thought to be another important site of entry of *E. ictaluri* into catfish. This is because, in acute ESC, lesions were first seen as infiltrations of macrophages (some of which containing engulfed bacteria) in the lamina propria and submucosa of the anterior intestine (Newton et al, 1989). The gill is also a primary site of *E. ictaluri* invasion. Nusbaum and Morrison (1996) demonstrated the colonization of the organism on the gill epithelium of channel catfish following immersion challenge. The internalized *E. ictaluri* into the host was able to resist phagocytosis and survive in the host phagocytic cells. Hence, it is believed that host phagocytic cells serve as a vehicle for the systemic dissemination of *E. ictaluri* (Morrison

and Plumb, 1994; Shotts et al, 1986).

7.4. Diagnostic Methods

Isolation of *E. ictaluri* from diseased fish is achieved from kidney, liver, spleen, intestine, brain and skin or muscle lesions by inoculation of material into brain heart infusion (BHI) agar, blood agar, or *Salmonella-Shigella* (SS) agar. Following incubation at 26°C for 48h, smooth circular (2 mm diameter), slightly convex, entire, non-pigmented colonies develop (Hawke 1979; Austin and Austin, 2007) . A selective medium, called *E. ictaluri* medium (EIM), has been formulated for the isolation of *E. ictaluri* (Shotts and Waltman, 1990).

7.4.1. Clinical Signs and Gross Pathology

Once channel catfish suffer ESC, the fish refuse to feed, show depigmentation of the skin, and swim with a spiral movement. Gross external lesions include haemorrhages around the mouth, on the lateral and ventral portions of the body, and on the fins. Other signs include pale gills, exophthalmia, and small ulcerations on the body (Bullock and Herman, 1985).

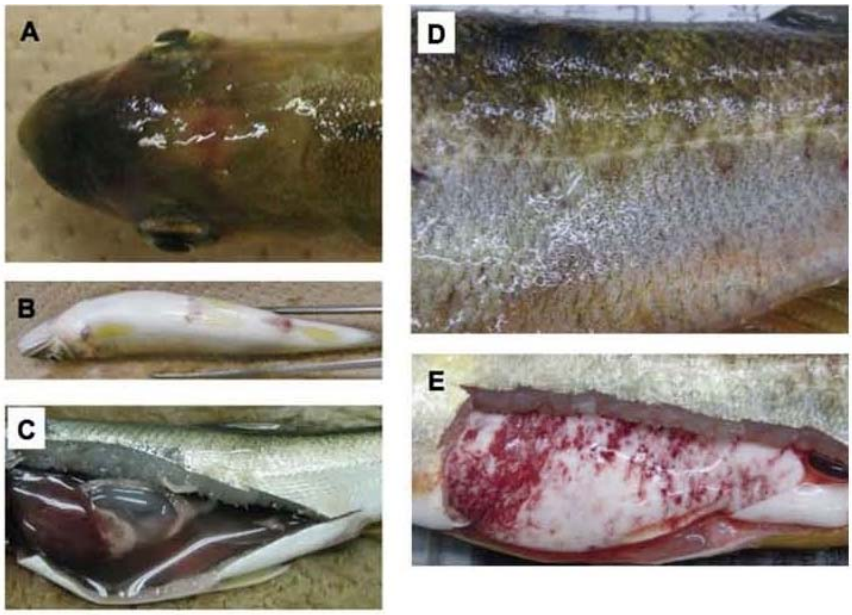

Figure 7.2. Clinical signs of *E. ictaluri*-infected ayu. Observation of exophthalmos (A), abdominal dissension and reddening of the anus (B), ascites (C), protruded lesion on the skin (D), and reddening of the gonad (E) were reported (Sakai et al, 2008). (All photographs by Dr. Sakai T., National Research Institute of Aquaculture, Fisheries Research Agency).

Acute ESC and chronic ESC in channel catfish were experimentally demonstrated (Shotts et al, 1986; Newton et al, 1989). Lesions compatible with acute ESC including cutaneous haemorrhage and ulceration, enteritis, olfactory sacculitis, hepatitis and dermatitis were most commonly seen. Accumulation of bloody serous fluid within the body cavity, congestion and haemorrhages in the liver were also observed. Chronic ESC

was often characterized by "hole in the head" lesions. Dorsocranial swelling and ulceration, granulomatous olfactory neuritis/perineuritis and meningoencephalitis involving the olfactory bulbs, olfactory tracts and brain were typical in chronic ESC. In the case of ayu, haemorrhagic ascites were suggested to be a pathognomonic sign of *E. ictaluri* infection. The onset of pericarditis was also observed in diseased ayu (Sakai et al, 2008) (Figure 7.2).

7.4.2. Histopathology

For internal clinical signs, the most severely damaged organs in *E. ictalrui*-infected catfish are the liver and spleen (Plumb, 1999). Congestion and apparent ellipsoids in the spleen are often observed in ESC-affected catfish. Necrosis of hepatocytes with accumulations of macrophages and neutrophils are observed throughout the liver in *E. ictaluri*-infected catfish. The macrophage in the tissues of the infected fish frequently contains intracellular bacteria (Shotts et al, 1986). Inflammatory responses in the loose connective tissue of the olfactory sac, olfactory nerve and the periphery of the olfactory bulb in channel catfish are seen when *E. ictaluri* are infected via the nares. Diffuse granulomatous response in the telencephalon or olfactory lobe of the brain occurs in the affected catfish of typical "hole in the head" lesions (Shotts et al, 1986).

7.4.3. Diagnosis by PCR and Serological Techniques

Bilodeau et al. (2003) developed a real-time PCR technique for *E. ictaluri* detection. The sensitivity of detection was determined to be as low as the equivalent of 2.5 cells in a DNA sample. A set of PCR primers, targeting the upstream region of the fimbrial gene cluster, successfully detected isolates of *E. ictaluri* from catfish and ayu (Sakai et al, 2009). Besides PCR-based techniques, the usefulness of monoclonal antibodies (MAb) for indirect fluorescent antibody (IFA) techniques for confirming clinical diagnosis of ESC was suggested (Ainsworth et al, 1986). Enzyme linked immunosorbent assay (ELISA) methods have also been developed to detect catfish antibodies to *E. ictaluri* (Waterstrat et al, 1989). This ELISA technique is useful to check whether the fish have been infected with *E. ictaluri*.

7.5. Control

7.5.1. Prevention

Minimizing stressors, preventing overcrowding, using proper feeds but not overfeeding, maintaining high water quality, and removing dead fish as soon as possible are important management practices to reduce the effect of ESC. Discontinuing feeding (e.g., skipping 1 or 2 days between feeding) may be a good management practice when ESC strikes. This practice has been adopted by many channel catfish farmers instead of feeding a medicated-diet (Evans et al, 2011; Plumb 1999).

7.5.2. Chemotherapy

In the USA, chemotherapy of ESC in channel catfish is achieved by feeding medicated-diets containing antibiotics. Three antibiotics have been approved for ESC

control of channel catfish. These are florfenicol (Aquaflor), oxytetracycline (Terramycin), sulfadimethoxine-ormetoprim (Romet-30). Florfenicol is fed at 10 mg/kg of fish/day for 10 days. Oxytetracycline is fed at 50–75 mg/kg of fish/day for 12–14 days. Sulfadimethoxine-ormetoprim is also fed at 50–75 mg/kg of fish/day for 5 days. The withdrawal periods for florfenicol, oxytetracycline and sulfadimethoxine-ormetoprim are 12, 21, and 3 days prior to harvest, respectively (Evans et al, 2011).

7.5.3. Vaccine

Saeed and Plumb (1986) demonstrated enhancement of the agglutination antibody titre and protection by multiple injections of the LPS prepared from *E. ictaluri.* Similarly, it is reported that an intraperitoneal injection of a whole-cell preparation of formalin-killed *E. ictaluri*, cellular extract, and crude membrane produced agglutination antibody titres (Vinitnantharat and Plumb, 1992). Primary immunodominant antigens in the cell membrane of *E. ictaluri* with a molecular mass of 36 kDa and 60 kDa provided protection to channel catfish (Vinitnantharat et al, 1993). Thune et al. (1994) investigated the practical application of vaccination against *E. ictaluri*. They demonstrated the potential of an oral/immersion method of killed *E. ictaluri* vaccine to small size channel catfish. Recently, because of its higher efficacy, attenuated *E. ictaluri* vaccine attracts more attention than other types of vaccines. Klesius and Shoemaker (1999) developed an attenuated vaccine strain RE-33 by passages on an antibiotic of refampicin. Strain RE-33 (AQUAVAC-ESC^{TM}) has been available since 2000 for catfish farming in the USA. About 25% of all catfish fry and/or fingerlings produced in south-eastern USA have been immunized with this attenuated vaccine (Evans et al, 2011)[5].

7.6. Recent Topics

The entire genome sequence of *E. ictaluri* 93-146 has been determined (Williams et al, 2012). Yang et al. (2012) also sequenced the whole genome of *E. ictaluri* ATCC33202 to conduct comparative phylogenomic analyses of *Edwardsiella* species. They found that 93-146 and ATCC33202 share most of the genomic islands (GIs) and the insertion sequence (IS) elements between themselves, but *E. tarda* strains have higher sequence divergence of GIs and IS elements. The conserved GIs and IS element profiles in *E. ictaluri* strains imply that the genomes of different *E. ictaluri* might be kept less modified in relatively fixed hosts, whilst the variance distribution of GIs and IS elements in different *E. tarda* strains may correspond to the broad host range properties. Presence of the gene clusters of the type III and type VI secretion systems was determined in the genome of *E. ictaluri*. The importance of these secretion systems in the virulence of *Edwardsiella* species was suggested from functional studies using gene mutagenesis techniques (Thune et al, 2007; Wang et al, 2009;Chakraborty et al, 2011; Rogge and Thune, 2011).

8. MOTILE AEROMONADS DISEASE

Tomokazu Takano

8.1. Synopsis

A haemorrhagic septicaemia caused by *Aeromonas hydrophila* complex has been

observed in numerous species of fresh water fish and occasionally in marine fish and amphibians, reptiles, cattle and humans through out the world. Especially, the severe diseases occur in cultured freshwater fish (Bullock *et al*., 1971; Aoki, 1974; Egusa, 1978; Khardori and Fainstein, 1988; Schäperclaus *et al*., 1992). In this section, characteristics, diagnostic methods, and control of *A. hydrophila* are reviewed.

8.2. Introduction

Infectious abdominal dropsy in common carp has been attributed to the *A. hydrophila* group (*Aeromonas punctata*) and was first described by Schäperclaus (1930). During the 1960s, outbreaks of red fin disease, caused by *A. hydrophila*, occurred frequently in cultured eel in Japan (Hoshina, 1962; Egusa, 1978). The bacterium inhabits widely in freshwater environment such as water and bottom sediments containing organic material, as well as in the intestinal tract of fish (Aoki, 1974; Egusa, 1978; Hazen *et al*., 1978; Seidler *et al*., 1980; Kaper *et al*., 1981; van der Kooij and Hijnen, 1988; Sugita *et al*., 1994; Dumontet *et al*., 1996). Concurrent infection by *Saprolegnia parasitica* in cultured Japanese eel was also reported (Egusa, 1978). Hence, *Aeromonas hydrophila* is typically recognized as an opportunistic pathogen or secondary invader (Austin and Austin, 1987). Infection by *A. hydropila* became known in most cultured fresh water fish species. Therefore, *A. hydropila* is economically important in fresh water farming.

8.3. Disease Agent

Characteristics

Kou (1972a, 1973) and Wakabayashi *et al*. (1981) recognized that almost all pathogenic strains of motile aeromonads relevant to aquaculture were encompassed within *A. hydrophila* biover. *hydrophila*, proposed by Popoff and Véron (1976).

Aeromonas hydrophila is a Gram-negative rod-shaped bacterium and is motile, due to a monotrichous polar flagellum. The bacterium measures 0.3–3.0 μm in diameter and 1.0–3.5 μm in length. It was no spore stage or capsule. The optimum growth temperature is 28°C, but growth can occur at 27°C. Colonies on nutrient agar are white to pale pink, round and convex, with entire margins.

The biochemical characteristics are shown in Table 8.1. It is a facultative anaerobe, fermenting carbohydrates to acid, or acid and gas. *Aeromonas hydrophila* is resistant to the vibriostatic agent O/129 (phosphate: 2,4-diamino-6,7-diisopropylpteridine phosphate) 150 μg, reduces nitrates to nitrate, is unable to grow in media containing 6.5% sodium chloride (NaCl) and is generally resistant to ampicillin and carbenicillin. The guanine plus cytosine (G + C) content of the deoxyribonucleic acid (DNA) is 57–63% (MacInnes *et al*., 1979; Aoki, 1999).

Table 1. Biological characteristics of *Aeromonas hydrophila.*

Characteristics	Response
Indole production in 1% peptone water	+
Aesculin hydrolysis	+
Growth in potassium cyanide (KCN) broth	+
L-Histidine and L-arginine utilization	+
L-Arabinose utilization	+
Acetoin from glucose (Voges-Proskauer (VP) reaction)	+
H_2S from cysteine	+
Oxidase	+
Cytochrome Oxidase	+
Catalase	+
Methyl red (MR) experiment	d
Acethylmethylcarbinol production	+
2,3-Butanediol production	+
2,3-Butanediol dehydrogenase	+
β-Galactosidase production	+
Phosphatase	+
Nitrate reduction	+
Urease	−
Malonate	−
Gelatin liquefaction	+
Casein digestion	+
Loeffler serum digestion	+
Starch hydrolysis	+
Lipase	+
Lecithinase	+
Glucuronate utilization	+
Ornithine decarboxylase	−
DNAse	+
RNAse	+
Haemolysis	+
Carbohydrate decomposition	
Adonitol	−
Aesculin	d
Arabinose	d
Cellobiose	d
Dextrin	+
Dulcitol	−
Fructose	+
Galactose	+
Glucose	+
Glycerol	+
Glycogen	+
Inositol	−
Insulin	−
Lactose	d
Maltose	+
Mannitol	+
Mannose	+
Melezitose	−
Raffinose	d
Rhamnose	d
Salicin	d
Sorbitol	d
Sorbose	−
Starch	+
Sucrose	d
Trehalose	+
Xylose	−

+, Typically positive; −, typically negative; d, differs among strains;
H_2S, hydrogen sulphide; DNAse, deoxyribonuclease; RNAse, ribonuclease.

Table 8.1. Biological characteristics of *Aeromonas hydrophila.*

Classification

Aeromonas hydrophila contains thermostable O, thermolabile K and flagellar H antigens. Serologically, the O antigen of *A. hydrophila* is hetero generous (Sakazaki and Shimada, 1984; Janda *et al*., 1994, 1996). Different serotypes have been observed from various sources of fish, isolated in different years and places (Eddy, 1960; Bullock, 1966). Interestingly, a common antigen has been found among virulent strains (Kou, 1972b; Leblanc *et al*., 1981). Protein fingerprints do not correlate with biochemical characteristics. Both phenotype and protein fingerprints show clustering of epizootiologically related isolates (Millership and Want, 1993). Maruvada *et al*. (1992) detected species-specific polypeptides of the outermembrane from *A. hydrophila*, and Wilcox *et al*. (1992) suggested that outermembrane protein profiles were useful for confirming the identity of *A. hydrophila*. Shaw and Hodder (1978) showed that O-polysaccharides were remarkably similar structure in motile Aeromonas species, including *A. hydrophila*. MacInnes *et al*. (1979) investigated the DNA homology of 17 strains of *A. hydrophila*, which had been collected from various sources, using *A. hydrophila* ATCC7966 as a reference strain. The percentage homology DNA ranged from 39 to 100%, with a mean value of 64.7%. *Aeromonas hydrophila* does not seem to show any significant divergence among the 17 strains investigated. The 16S ribosomal DNA (rDNA) from ten species of *Aeromonas* was sequenced to analyze relatedness (Martinez-Murcia *et al*., 1992). Homology for 16S rDNA of the ten species exhibited very high levels, ranging 98 to 100%. East and Collins (1993) showed that region encoding 23S ribonucleic acid (RNA) from *A. hydrophila* was identical to that of gamma division of Proteobacteria, *Escherichia coli* and *Plesiomonas shigelloides*. Small-subunit ribosomal RNA (rRNA) sequences of *Aeromonas* were examined for a phylogenetic analysis (Ruimy *et al*. 1994).

Host range and Pathogenesis

Most cultured fresh water fish are susceptible to infection by *A. hydropila*, such as brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), ayu (*Plecoglossus altivelis*), carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), clariid catfish (*Clarias batrachus*), Japanese eel (*Anguilla japonica*), American eel (*Anguilla rostrata*), gizzard shad (*Dorosoma cepedianum*), goldfish (*Carassius auratus*), golden shiner (*Notemigonus crysoleucas*) and tilapia (*Tilapia nilotica*) (Bullock *et al*., 1971; Egusa, 1978: Saitanu, 1986; Aoki, 1999).

A variety of possible virulence factors of *A. hydrophila* have been suggested, including lipopolysaccharides (endotoxins), extracelluar products (ECP), siderophores, the abilitu of attachment to host cells and surface proteins. The ECP include a cytotoxin, enterotoxin, haemolysins, protease, haemagglutinin and acetyl cholinesterase (Cahill, 1990; Gosling, 1996; Howards *et al*., 1996). *Aeromonas hydrophila* enters through the epithelium of the intestinal tract of fish. Enterotoxins of *A. hydrophila* cause fluid to accumulate in ligated rabbit ileal loops. Enterotoxins are divided into two types, cytotonic and cytotoxic.

8.4. Diagnostic Methods

Isolation

Aeromonas hydrophila can be grown on brain-heart infusion medium, tryptosoy agar,

neutrient agar and MacConkey agar with incubation at 20–25°C for 24–48 h. Numerous selective media have been developed fro the isolation and presumptive identification of *A. hydrophila* or motile aeromonads (Moyer, 1996), including Rimler-Shotts medium (Shotts and Rimler, 1973), modified peptone beef-extract glycogen agar (McCoy and Seidler, 1973), Rippey-Cabelli (membrane filter method (mA)) agar (Rippey and Cabelli, 1979), MacConkey's agar supplemented with trehalose (Kaper *et al*., 1979) and starch-ampicillin agar (Palumbo *et al*., 1985). Davis and Sizemore (1981) reported that Rimler and Shotts medium and Rippey-Cabelli agar were not suitable for *A. hydrophila.* Arcos *et al*. (1988) compared six media for selective isolation of *A. hydrophila* and showed that mA agar gave the best recovery rate and also an acceptable specificity, but its selectivity was low. An API-20E test strip can be used for identification of the Enterobacteriaceae including *A. hydrophila*.

Clinical signs, Gross pathology and Histopathology

Diseased fish usually display cutaneous haemorrhage of the fins and trunk, and the condition is often referred to as 'red fin disease' (Hoshina, 1962). The bacteria multiply inside the intestine, causing a haemorrhagic mucousdesquamative catarrh. Toxic metabolites of *A. hydrophila* are absorbed from the intestine and induce a toxaemia. Capillary haemorrhage occurs in the dermis of fins and trunk and in the submucosa of the stomach. Hepatic cells and epithelia of renal tubules show degeneration. Glomeruli are destroyed and the tissue becomes haemorrhagic, with exudates of serum and fibrin (Miyazaki and Jo, 1985; Miyazaki and Kaige, 1985). European carp infected with *A. hydrophila* show severe tail and fin rot and visible haemorrhage and ulceration of the body surface. Widespread proliferation of bacteria is usually observed in the intestine. In some reports (Fijan, 1972; Wolf, 1988), the histopathological phenomena associated with the rhabdovirus infection haemorrhagic septicaemia of carp have been erroneously attributed to motile aeromonads (Bullock *et al*., 1971).

Diagnosis by serological and molecular techniques

Aeromonas hydrophila has been identified by the gel-diffusion technique (Bullock, 1966), direct fluorescent antibody technique (Lewis and Allison, 1971), indirect fluorescent antibody technique (Lewis and Savage, 1972), immunoblotted sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Mulla and Millership, 1993). However, these methods are of limited value, because many different serological types of *A. hydrophila* are distributed in fish farms (Eddy, 1960; Bullock, 1966). Deoxyribonucleic acid probe hybridization technology is available for the direct detection and identification of mycroorganisms. However, many common DNA fragments between *A. hydrophila* and *A. salmonicida* were reported by Miyata *et al*. (1995). Therefore, it is unlikely that this hybridization technique will be successful for this species. Cascón *et al*. (1996) found a specific PCR primer set for the detection of *A. hydrophila* hybridization group 1.

8.5. Control

Prevention

Out breaks of the disease are usually associated with a change in environmental conditions. Stressors, including overcrowding, high temperature, a sudden change of temperature, rough handling, transfer of fish, low dissolved oxygen, poor nutritional

status and fungal or parasitic infection, contribute to physiological changes and heighten susceptibility to infection. *Aeromonas hydrophila* is widely distributed in the intestinal tract of cultured fish and the water and sediments of fresh water ponds which are rich in organic materials. Virulent strains of *A. hydrophila* in these environments are possible sources of infection. Therefore, minimizing stressors, preventing overcrowding, using proper feeds, maintaining clean environment of ponds are important management practices to reduce *A. hudrophila* infection (Aoki, 1999; Cipriano and Austin, 2011).

Chemotherapy

Chemotherapeutic agents are used for the treatment of *A. hydrophila* in fish farms (Aoki, 1992). Isolates of *A. hydrophila* have been found to be sensitive to chloramphenicol, florfenicol, tetracycline, sulphonamide, nitrofuran derivatives and pyridonecarboxylic acids (Aoki and Egusa, 1971; Endo *et al*., 1973; Katae *et al*., 1979; Fukui *et al*., 1987). Fluorinated analogue, tetracycline derivatives, nitrofuran derivatives, sulphonamide and pyrodonecarboxylic acids are effective in oral treatments (Austin and Austin, 1987).

Vaccine

Experimental vaccination for prophylaxis against infection of *A. hydrophila* has been examined (Stevenson, 1988). Fish immunized either intramuscularly or intraperitoneally with vaccine showed protection against challenge. The agglutinating antibody titre increased in the serum of immunized fish (Song *et al*., 1976; Ruangpan *et al*., 1986; Karunasagar *et al*., 1991). Immersion vaccination of channel catfish using polyvalent sonicated antigens of *A. hydrophila* provides protection (Thune and Plumb, 1982). Lamers *et al*. (1985) noted that agglutinating antibody was recognized in the serum of carp immunized with *A. hydrophila* bacterin, following a second immersion with this vaccine. Catfish immunized intraperitoneally by injection with the acid extract of the S-layer protein of *A. hydrophila* were protected from the homologus, virulent strain (Ford and Thune, 1992). Serological types of *A. hydrophila* are heterogeneous and polyvalent vaccine is thought to be necessary for prevention of the infection.

8.6. Recent Topics

Draft genome sequence of *A. hydrophila* SNUFPC-A8 which was isolated from a kidney of a moribund cherry salmon (Oncorhynchus masou masou) became available. The sequence data were assembled into 59 contigs and 300 singletons. The draft genome of *A. hydrophila* SNUFPC-A8 was 4,969,090 bp in length, and a total of 4,779 open reading flames were discovered (Han *et al*., 2013). A systematic analysis of the virulence factors based on this genome database will uncover the details of the disease caused by *A. hydrophila.*

Glossary

NaCl : Sodium chloride,

DNA : Deoxyribonucleic acid,

G + C : Guanine plus cytosine,

rDNA : Ribosomal DNA,

ECP : Extracelluar products,

SDS-PAGE : Sodium dodecylsulphate-polyacrylamide gel electrophoresis

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