# [4] Diseases Caused By Bacterial Pathogens in Saltwater

## 7. EDWARDSIELLOSIS (EDWARDSIELLA TARDA)

*Tomokazu Takano*

### 7.1. Synopsis

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

### 7.2. Introduction

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

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

### 7.3. Disease Agent

#### 7.3.1 Characteristics

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

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

### 7.3.2 Genome Size

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

### 7.3.3 Serological Classification

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

### 7.3.4 Molecular Classification

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

### 7.3.5 Pathogenesis

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

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

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

### 7.4. Diagnostic Methods

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

#### 7.4.1 Clinical Signs and Gross Pathology

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

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

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

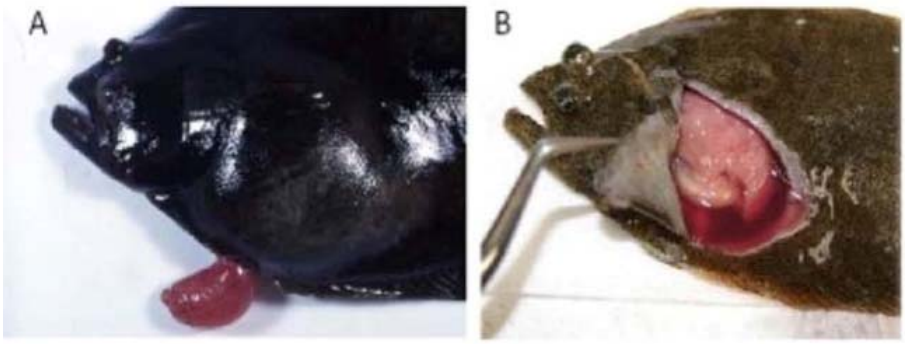


Figure 7.1. Japanese flounder infected with *Edwardsiella tarda*. A swollen abdomen and prolapsed rectum due to intensive ascites are seen frequently (A) (Photograph by Dr. Fukuda Y., Oita Prefectural Agriculture, Forestry and Fisheries Research Center). Accumulation of bloody ascetic fluid, abscess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

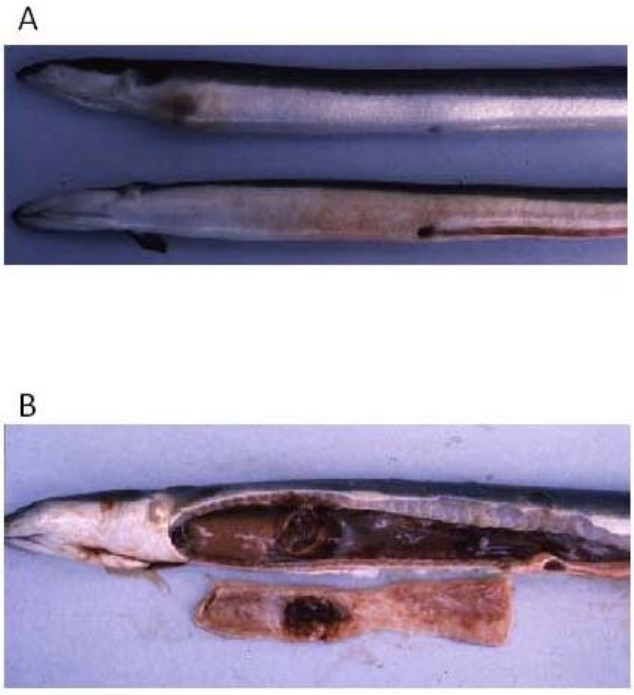


Figure 7.2. Japanese eel infected with *Edwardsiella tarda*. The upper fish has a swollen abdomen due to ulceration of the liver. The lower fish has haemorrhaging in the skin and the congested anal fin (A). The liver in diseased eel collapses due to extreme ulceration (B) (All photographs by Dr. Miyakawa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

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

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

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

### 7.4.2. Histopathology

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

### 7.4.3. Diagnosis by PCR and Serological Techniques

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

## 7.5. Control

### 7.5.1 Prevention

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

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

### 7.5.2 Chemotherapy

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

### 7.5.3 Vaccine

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

## 7.6. Recent Topics

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

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

**Glossary**

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

## 8. BACTERIAL HEMOLYTIC JAUNDICE

*Takaji Iida*

### 8.1. Synopsis

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

### 8.2. Introduction

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