[3] Diseases Caused By Bacterial Pathogens In Inland Water

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).

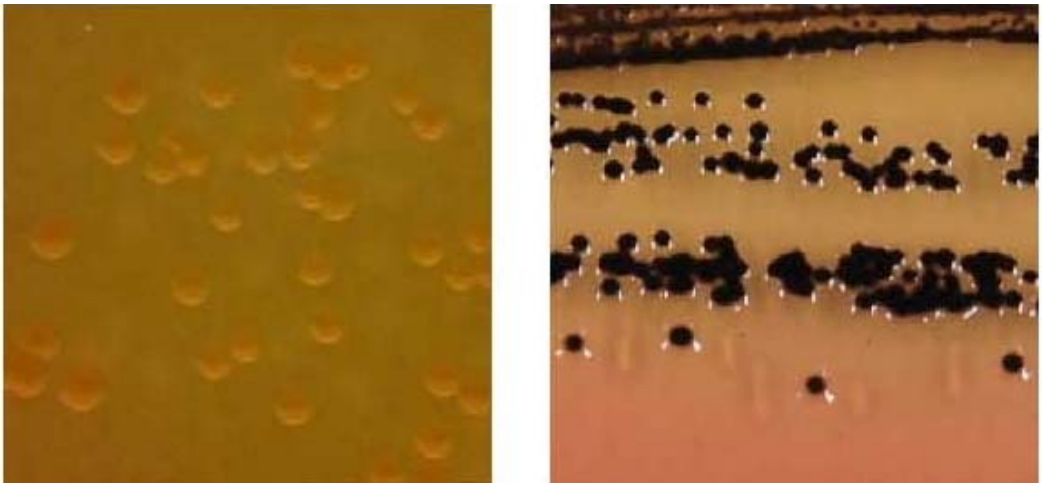

Figure 7.1. Colonies of *E. ictaluri* and *E. tarda* on SS agar. *Salmonella-Shigella* agar permits detection of hydrogen sulfide (H_2S) by the production of colonies with black centers. *Edwardsiella ictaluri* isolated from ayu does not produce H_2S (A), whilst *E. tarda* isolated from Japanese flounder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

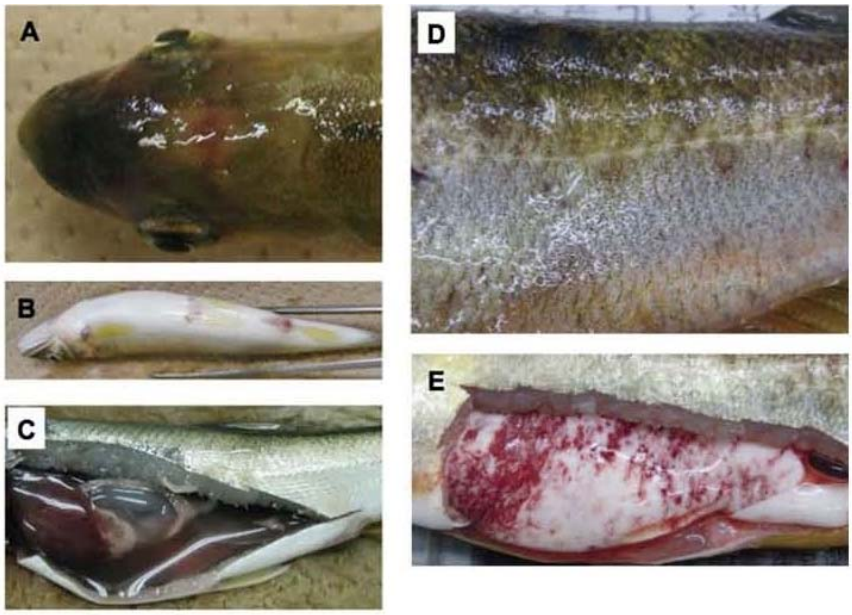

Figure 7.2. Clinical signs of *E. ictaluri*-infected ayu. Observation of exophthalmos (A), abdominal dissension and reddening of the anus (B), ascites (C), protruded lesion on the skin (D), and reddening of the gonad (E) were reported (Sakai et al, 2008). (All photographs by Dr. Sakai T., National Research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