[4] Diseases Caused By Bacterial Pathogens in Saltwater

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

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

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

3.6. Recent Topics

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

Glossary

PCR: Polymerase chain reaction,

FRET assays: Fluorescence/förster resonance energy transfer

4. PASTEURELLOSIS

Tae-Sung Jung and Takashi Aoki

4.1. Synopsis

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

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

4.2. Introduction

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

4.3. Characteristics of the Disease Agent

4.3.1 Characteristics

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

4.3.2 Genome Size

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

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

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

4.3.3 Serological Classification

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

4.3.4 Molecular Classification

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

4.3.5 Pathogenesis

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

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

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

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

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

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

4.4. Diagnosis

4.4.1 Clinical Signs

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

4.4.2 Gross pathology

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

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

4.4.3 Histopathology

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

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

4.5. Diagnostic Methods

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

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

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

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

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

4.6. Prevention and Control

4.6.1 Prevention

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

4.6.2 Chemotherapy

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

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

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

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

4.6.3 Vaccine

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

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

4.7. Recent Topics

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

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

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

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

Glossary

ECPs : Extracellular products,

ELISA : Enzyme-linked immunosorbent assay,

PTE : Potassium thiocyanate extract,

NB : Naked bacteria

5. VIBRIOSIS

Yukinori Takahashi and Jun-ichi Hikima

5.1. Synopsis

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

5.2. Introduction

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