[4] Diseases Caused By Bacterial Pathogens in Saltwater

(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

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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

have been recognized among a variety of fish in the world (Hoshina, 1957; Muroga, 2004). *V. anguillarum*, *V. ordalii* and *V. salmonicida* are designated from closely related strains. *V. ordalii* appear to be the seriously pathogenic against salmonids, ayu sweetfish (*Plecoglossus altivelis altivelis*) and jacopever (*Sebastes schlgelii*) (Muroga, 2004; Schiewe et al, 1981). *V. salmonicida* was isolated from salmonid and the other fish such a cod in cold water (Hjeltnes and Roberts, 1993; Schiewe et al, 1981). The organisms of all serotypes are more pathogenic in salmonids than in the other fishes. *V. vulnificus* was the first to isolated from Japanese eel (Muroga, 2004; Egidius et al, 1986). The bacteria were also reported to be pathogenic to European eel and human.

V. alginolyticus is an opportunistic pathogen or serves as a secondary invader of traumatized fish. *V. parahaemolyticus*, known to be a bacterium involved in food poisoning, was isolated from cultured yellowtail (*Seriola quinqueradiata*) (Muroga, 2004; Colorni et al, 1981). Vibriosis caused by *V. penaeicida* in cultured shrimp has been observed since 1980, and was known to have caused considerable loses to shrimp farms in Japan and New Caledonia (Hatai et al, 1975; Takahashi et al, 1985 ; Ishimaru et al, 1995 ; Costa et al, 1998).

5.3. Disease Agent

5.3.1. Characteristics

Table 5.1 shows the biological and biochemical characteristics of *Vibrio* species pathogenic to fish. The species consists of Gram-negative straight or straightly curved rods (Figure 5.1). They are non spore-forming and motile by monotrichous or multitrichous. Metabolism is aerobic or facultatively anaerobic and carbohydrates are fermented with the production of acid but not gas.

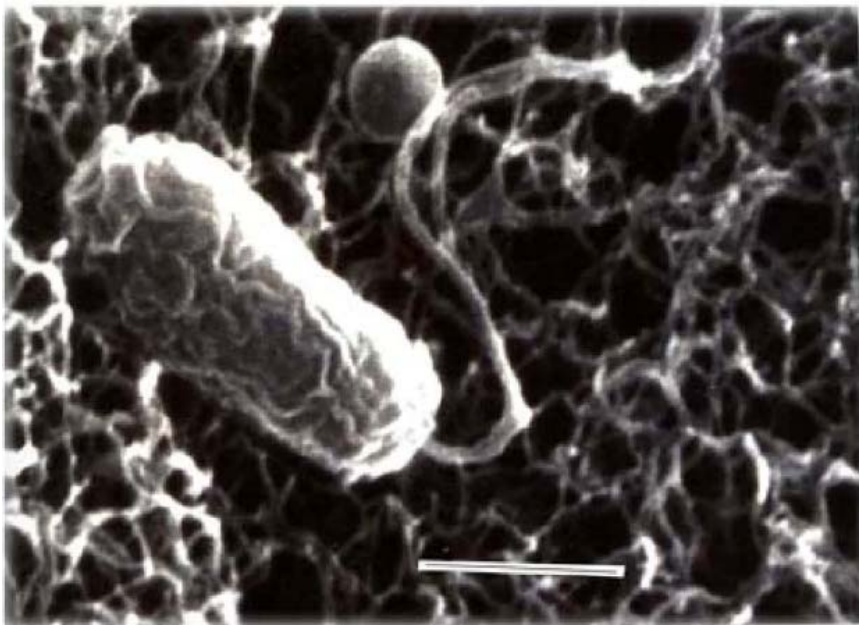

Figure 5.1. Electron microscopic photograph of *Vibrio anguillarum* (scale bar; 1.0 μm).

V. anguillarum typically grows within 24h at 25 ^{o}C on nutrient agar or TSA as a circular, raised, yellow-brown, opaque colony of 2 to 4mm. Growth on the media was observed at 10 to 35^{o}C with NaCl concentrations of 0.5 to 6.0 or 7.0%. No growth was observed in the media with 8.0% NaCL concentration.

This bacterium gives positive Voges-Proskauer reaction, gelatin liquefaction, nitrate reduction arginine hydrolysis, utilization of sucrose, and does not produce lysine, ornithine decarboxylase. The mol% G+C of the DNA is 44-46 (Muroga, 2004).

Characteristics	***Vibrio anguillarum***	***V. parahaemolyticus***	***V. alginolyticus***	***V. ordalii***	***V. salmonicida***	***V. vulnificus***	***V. penaeicida***
Swarming	-	-	+	-	-	-	-
Grows at 35^{o}C	+	+	+	-	-	+	-
Grows at 40^{o}C	-	+	+	-	-	-	-
NaCl tolerance: 6%	+	+	+	+	-	-	-
NaCl tolerance: 8%	-	+	+	-	-	-	-
Gas from glucose	-	-	-	-	-	-	-
VP reaction	+	-	+	-	-	-	-
Gelatinase	+	+	+	+	-	+	+
Nitrate reduction	+	+	+	+	-	+	+
Arginine hydrolysis	+	-	-	-	-	-	-
Lysine decarboxilization	-	+	+	-	ND*	+	-
Ornithine decarboxilization	-	+	+	-	ND*	+	-
Utilize sucrose	+	-	+	+	-	-	-
DNA G+C mol%	44~46	46~47	46~47	43~44	42	46~48	46.2~47.0

Table 5.1. Biological and biochemical characteristics of fish pathogenic *Vibrio* species.

V. ordalii grows after 4 to 6 days at 22 on TSA as a white, circular, convex, translucent colony of about 1-2mm. Growth on the media is observed at 15-30^{o}C and NaCl concentrations of 0.5-5.0 or 6.0% (Schiewe et al, 1981). This bacterium gives positive gelatin liquefaction, nitrate reduction, utilization of sucrose and did not produce arginine hydrolase, lysine, orninthine decarboxylase (Schiewe et al, 1981). The mol% G+C of the DNA is 43-44 (Schiewe et al, 1981).

V. salmonicida has low growth temperature, 1-22^{o}C with an optimum of 15^{o}C. Some distinguishing features of the organisms are negative Voges-Proskauer reaction, gelatin liquefaction, nitrate reduction, arginine hydrolysis, lysine, ornithine decarboxilization, utilization of sucrose (Schiewe et al, 1981). The mol% G+C of the DNA is 42.

V. vulnificus grows rapidly within 24h at 25°C on nutrient agar as a circular, raised colony of 3 to 5mm. It is observed to grow in temperatures from 18 to 39°C and media with NaCl concentrations of 0.1 to 4.0%. This bacteria displays negative Voges-Proskauer reaction, arginine hydrolysis and utilization of sucrose (Egidius et al, 1986; Nishibuchi et al, 1979).The mol% G+C of the DNA is 46-48.

V. alginolyticus swarms grow on nutrient agar, TSA and BHI media. Growth of *V. alginolyticus* and *V. parahaemolyticus* are observed at 10 to 40°C. Growth occurred in media with NaCl concentrations of 0.5 to 7.0 or 8.0%. *V. alginolyticus* gives positive Voges-Proskauer reaction, gelatin liquefaction, nitrate reduction, lysine, ornithin decarboxilization, utilization of sucrose and do not produce arginine hydrolase (Hjeltnes and Roberts, 1993; Muroga, 2004). The mol% G+C of the DNA is 46-47. *V. parahaemolyticus* displays negative Voges-Proskauer reaction, arginine hydrolysis and utilization of sucrose (Muroga, 2004).

Shrimp pathogenic *V. penaeicida* grows within 24 to 48h at 25°C on ZoBell's agar as a circular, raised, whitish opaque colony of 0.5 to 1.0mm. Growth on the media is observed at 10 to 30°C with an optimum of 25°C and NaCl concentrations of 1.5 to 4 or 5%. The bacterium gives positive gelatin liquefaction, nitrate reduction and do not produce arginine hydrolase, lysine, ornithine decarboxylase (Hatai et al, 1975; Takahashi et al, 1985). The mol% G+C of the DNA is 46.2-47,0 (Takahashi et al, 1985).

5.3.2. Serological Classification

The protein and lipopolysaccharides in the membrane of *V. anguillarum* have been extensively characterized in the eight (J-0-1~J-0-8) or sixteen (01~016) different O serogroups identified among Japanese strains or the pathogenic strain from marine fish (Saulnier et al, 2000 ; Tajima et al, 1985).

The Serological types of *V. ordalii* are in accord with the serotype J-0-1 of *V.anguillarum* (Muroga, 2004). The organisms strains showed 58 to 69% relatedness to *V. anguillarum* by analysis of DNA-DNA hybridizations (Schiewe et al, 1981).

V. salmonicida have two distinct serotypes: salmonids or the other fish pathogenic strains. The organisms was distinguished from *V. anguillarum* and *V. ordalii* by DNA-DNA hybridization (Grisez and Ollevier, 1995).

5.3.3. Genome Sequence

To date, the complete gemone of only one species, *V. anguillarum* 775 strain (isolate from coho salmon *Onchorhynchus kisutch*; ATCC 68554), has been determined among *Vibrio* species.[22)] Meanwhile, draft sequences of *V. anguillarum* 96F strain (isolate from striped bass, *Morone saxatilis*), RV22 (isolate from turbot, *Scophthalmus maxinus*) and *V. ordalii* ATCC 335509 (isolate from coho salmon) are also published in GenBank genome database (GenBank Assembly IDs: GCA_000217675.1, GCA_000257165.1, GCA_000257185.1, GCF_000257205.1).

The complete genome sequence of *V. anguillarum* 775 consists of two chromosomes,

3,063,912 bp chromosome 1 (Chr1) and 988,135 bp chromosome 2 (Chr2), in addition to the 65,009 bp pJM1 plasmid. Chr1 and Chr2 contain 2,864 and 951 annotated genes, respectively. The majority of genes for essential cell functions and pathogenicity are located on Chr1. In constract, Chr2 contains a larger fraction (59%) of hypothetical genes than does Chr1 (42%), and also harbors a superintegron, as well as host addiction genes that are typically found in plasmids (Wiik and Egidius, 1986).

5.3.4. Pathogenesis

The extracellular protease of *V. anguillarum* was demonstrated as a factor of the severe focal myonecrosis and liquefaction of fish musculature (Naka et al, 2011; Stensvåg et al, 1993). The pathogenesis of the fish pathogenic *Vibrio* to produce hemolytic anemia, which results in high circulating and melano-macrophage related iron levels in affected fish, is related to its high requirement for iron. The pathogenic strains have a well-developed iron sequestering mechanism based on secretion of a siderophore, which induces separation of plasma and tissue iron from its transferrin or ferritin binding proteins. They form complexes to the siderophore and attaches to specific transport outer cell membrane proteins for absorption into the bacterial cells (Morita et al, 1996; Roberts, 1975).

5.3.5 Virulence Factors and the Coding Genes

V. anguillarum virulence is associated with the presence of a plasmid-mediated iron uptake system, which is involved in an efficient iron-sequestering system consisting of the indigenous siderophore anguibactin (Actis et al, 1985; López and Crosa, 2007). In *V. anguillarum, angB/G, angM, angN, angR* and *angT* genes, which code for non-ribosomal peptide synthetasea (NRPSs), catalyse the synthesis of anguibactin (Actis et al, 2011). In addition, the genes *fatA, fatB, fatC* and *fatD* are involved in the transport of ferric-anguibactin complexes. These transport genes, together with the biosynthesis genes *angR* and *angT*, are included in the iron-transport biosynthesis (ITB) operon (López and Crosa, 2007).

Other virulence factors in *Vibrio* species such as heamolysin, flagellum and heamin-uptaking factors have been known. Haemolysin is a protein that causes lysis of red blood cells by damaging their cell membranes. In *V. anguillarum*, there are four heamolysin genes: vah2, vah3, vah4 and vah5. These ORFs encode 291, 690, 200 and 585 amino acid residues, and were found to be 33, 75, 22 and 66 kDa in size, respectively. Homologues of these genes were also found in *V. vulnificus* and *V. cholerea* (López and Crosa, 2007).

Flagellum is also involved in the virulence of *V. anguillarum* (Di Lorenzo et al, 2003). The product of flagellin gene (*flaA*) is needed for crossing the fish integument and might play a role in virulence (Chart, 1983). The genes, *virA* and *virB* encoding a major surface antigen are important for the virulence of *V. anguillarum* (Milton et al, 1996). This surface antigen appears to be a lipopolysaccharide located on the outer sheath of the flagellum and is expressed *in vivo* during fish infections with the flagellum. Another gene, *virC* is also essential for virulence although the function of the product is still unknown (Norqvist and Wolf-Watz, 1993).

V. anguillarum can utilize heamin and heamoglobin as iron sources. Nine genes, *huvA*, *huvZ*, *huvX*, *tonB1*, *exbB1*, *exbD1*, *huvB*, *huvC* and *huvD*, encoding the proteins involved in heamin transport and utilization, are clustered in a 10-kb region of chromosomal DNA (Milton et al, 1995; Mouriño et al, 2004).

5.4. Diagnostic Methods

5.4.1 Clinical and Histopathological Signs

In juvenile fish, first signs of the vibriosis are often anorexia, abrasion on the skin or fins, darkening and sudden death. In young and adult stages, first signs of losses are erosious leukoplakia, haemorrhage on the skin and fins (Figure 5.2). The developmental symptoms are haemorrhagic ulcers on the mouth, head, trunk skin or else local necrotic lesions in the muscle (Figure 5.3) (Hjeltnes and Roberts, 1993; Muroga, 2004; Mouriño et al, 2006; Muroga, 1992). Other symptoms like exophthalmus, haemorrhage of periorbitis, reddening and expansion of anus are observed.

Figure 5.2. Ayu sweetfish infected with vibriosis caused by *Vibrio anguillarum*, showing the haemorrhage on the skin.

Figure 5.3. Yellowtail infected with vibriosis caused by *Vibrio anguillarum*, showing skin ulcer.

Internally, the main signs such as intestinal haemorrhage and congestion of the liver, spleen and kidney are seen in affected fish especially at progressed stages (Hjeltnes and Roberts, 1993; Muroga, 2004).

Histopathological signs are characterized by the skin lesions which comprise hypodermal inflammatory foci extending deep into the muscle. There is severe myofibrillar necrosis with the centre of the lesion comprising an agglomerate of sacroplasmic debris. There are local necrosis in the liver, spleen and kidney a depletion and necrosis of haemopoietic elements (Hjeltnes and Roberts, 1993; Muroga, 2004).

The typical symptoms of vibriosis by *V. penaeicida* in diseased shrimp are cloudines of the muscle at the 6th abdominal segment and brown spots in the gills and lymphoid organ (Figure 5.4) (Hatai et al, 1975). Histopathological characteristics include extensive necrosis caused by severe bacterial invasion and multiple formation of melanized nodules in the lymphoid organ (Kusuda and Salati, 1993).

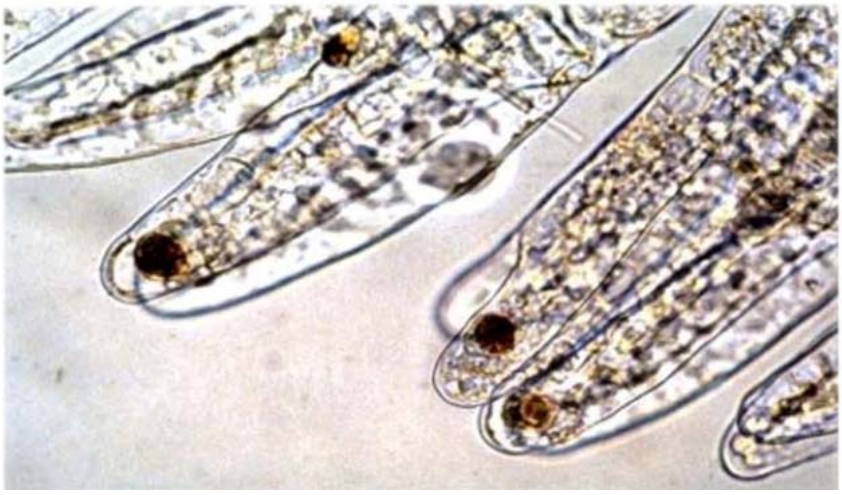

Figure 5.4. Shrimp infected with vibriosis caused by *Vibrio penaeicida*, showing brown spots in the gills.

5.4.2 Diagnosis

(2-1) Slide agglutination test

The rapid diagnostic test is usually performed to diagnose suspected culture of bacteria isolated in artificial culture. . One colony of a fresh culture of the organism is mixed with saline on a degreased glass slide in two separate drops. A drop of prepared anti-many serogroups of *Vibrio* species rabbit antiserum is added to one drop and a drop of saline to the other.

(2-2) Fluorescent antibody method

The method has advantages for the experienced researcher in rapid diagnosis of vibriosis.

Smears or crystat sections of suspected tissue are fixed in acetone for 5min, the washed in phosphate buffered saline (PBS). A drop of prepared anti–many serogroups of *Vibrio* species rabbit antiserum or the immunoglobulin G (IgG) is added on the tissue and

incubated at room temperature for 30 min. The antiserum (IgG) is washed off carefully with PBS and the slide washed three times with PBS. After air, drying a drop of fluorescein isothiocyanate labelled goat antiserum against rabbit globulin is added on the tissue, incubated for 30 min then washed with PBS. After several further washings the specimen is ready for examination.

(2-3) DNA hybridization method using a specific DNA probe

Diagnostic method	***Vibrio* species**	**Target gene**	**Reference**
Colony hybridization	*Vibrio anguillarum* (serotype A)	Chromosomal DNA (Randamly cloned DNA fragment, 562 bp)	32
	V. anguillarum, V. ordalii	5S rRNA gene (synthesized oligonucleotide)	44
Southern blot hybridization	*V. anguillarum*	16 and 23S rRNA gene (derived from *Escherichia coli;* merchandised by Boehringer Mannheim)	29
	V. anguillarum (serotype O1)	16 and 23S rRNA gene (derived from *Escherichia coli;* merchandised by Boehringer Mannheim)	30
	V. anguillarum	16 and 23S rRNA gene (derived from *Escherichia coli;* merchandised by Boehringer Mannheim)	31
PCR	*V. anguillarum*	Hemolysin gene (PCR products: 490 bp)	33
	V. anguillarum	*rpoS* gene (PCR products: 689 bp)	34
	V. anguillarum	*empA* gene (PCR products: 439 bp)	35
	V. anguillarum	*toxR* gene (PCR products: 93 bp)	36
	V. anguillarum	16S rDNA (PCR products: 81 bp)	36
	V. trachuri	Chromosomal DNA (Randamly cloned spcific region, 417 bp)	45
	V. vulunificu	Cytotoxin-hemolysin gene (PCR products: 519 bp)	37
	V. vulunificu	23S rRNA gene (PCR products: 978 bp)	39
	V. vulunificu	Cytolysin-hemolysin gene (PCR products: 1416 bp)	38
LAMP	*V. anguillarum*	metalloproteinase (*empA*) gene	41
	V. alginolyticus	gyrase B (*gyrB*) gene	42
	V. nigripulchritudo	Intergenic spacer region (ITS)	43

Table 5.2. Diagnostic methods for detecting *Vibrio* species in infected fish.

The basic principle of the DNA hybridization is the binding/hybridization of a single stranded DNA with its complementary DNA thereby forming a double stranded DNA. It is can be used to distinguish close species because the probe DNA labeled with a radioactive isotope or a fluorescent substance specifically hybridized with the nucleotide sequence encoding a target gene. In the DNA hybridization, there are three kinds of methods: colony hybridization, dot-blot hybridization and Sothern blot hybridization. The bacterial DNA fixed on a cellulose-nitrate or nylon membranes, and the labeled DNA probe is made to react in a hybridization solution, and then the radioactive isotope or fluorescent substance labeling the probe are detected by exposure on an X-ray film after washing.

Vibrio species detected using the DNA hybridization method have been reported (Table 5.2). *V. anguillarum* was detected by Southern bolt hybridization using 16S and 23S rRNA gene probe derived from *Escherichia coli*, which was commercialized by Boehringer Mannheim (Egusa et al, 1988; Pedersen et al, 1994). *V. anguillarum* serotype A was distinguished by colony hybridization using DNA probe encoding the serotype-specific gene (Skov et al, 1995).

(2-4) Polymerase chain reaction (PCR) method

PCR is a technology developed using the Taq DNA polymerase capable of working effectively in high temperatures (a heat-stable DNA polymerase) and allows for the amplification of a target gene. This method is described in detail in *Diagnosis-PCR Detection*. Because of its simplicity, quickness, and relatively higher accuracy compared with probe diagnostic methods, the PCR method is widely used for detection of specific genes of pathogenic bacteria. This method is also currently used to detect the causative bacteria for many fish and shellfish species.

PCR detection methods for Vibriosis have already been developed and used to successfully diagnose the presence of the causative agents of the disease. *V. anguillarum*, *V. trachuri*, and *V. vulunificu* have been detected by the PCR method (Table 5.2). Accurate and reliable diagnoses are made possible by amplifying genes that are specific to the bacteria such as hemolysin gene (Aoki et al, 1989), *rpoS* (Hirono et al, 1996), *empA* (Kim et al, 2008), *toxR* (Xiao et al, 2009), Cytotoxin-hemolysin gene (Crisafi et al, 2011; Hill et al, 1991) and 16S and 23S rRNA (Xiao et al, 2009; Coleman et al, 1996) (Table 5.2).

(2-5) Loop-mediated Isothermal Amplification (LAMP) method

The loop-mediated isothermal amplification (LAMP) reaction is an autocycling strand displacement DNA synthesis performed using a DNA polymerase with a high level of strand displacement activity and a set of specially designed inner and outer primers (Arias et al, 1995). The details of the principle are shown in "*Diagnosis: LAMP methods*".

The LAMP method has the following advantages when compared to the conventional PCR method: (1) since isothermal amplification of DNA is possible, this method does not need a thermal cycler; (2) the use of four different primers enhances its specificity; (3) the amplification efficiency of DNA is high (it amplifies 10^9 to 10^{10} times from 15 minutes in 1 hour); (4) the result can be visible without using electrophoresis (the LAMP products can be observed by adding pyrophoric acid magnesium into the reaction causing positive

samples to turn cloudy).

Target in genes in various Vibrio species including, *empA* in *V. anguillarum* (Notomi et al, 2000), *gyrB* in *V. alginolyticus* (Hongwei et al, 2010), and ITS (intergenic spacer region between 16S and 23S rRNA genes) in *V. nigripulchritudo* (Cai et al, 2010) were detected by LAMP method (Table 5.2).

5.5. Control

The prevention of vibriosis is best achieved by maintenance of environment, good husbandry and low stocking densities. However, in most cases such as clinical outbreaks, antibacterial agents are used for therapy. Oxytetracycline (OTC), sulphonamides (SA) or oxolinic acid (OA) are the most commonly used drugs. Oral-administration of OTC, SA or OA are used, with prescribed dosage of 50, 100-200, 20-30mg/kg fish 1day, respectively in for 5 days.

All of the licensed vaccines available are highly effective against salmonids vibriosis, which includes both *V. anguillarum* and *V. ordalii* infections (Hjeltnes and Roberts, 1993). In Norway, *V. anguillarum* and *V. salmonicida* combined vaccine is also available against salmonids (Hjeltnes and Roberts, 1993).

It has been reported that the inactivatied *V. anguillarum* vaccine is effective against infected ayu and salmonid fish by immersion method (Ellis, 1988; Mughal et al, 1986).In Japan, the immersion vaccination against salmonids, ayu sweetfish and yellowtail vibriosis are authorized by the government. As an antigen of the *Vibrio* vaccine, freezed-killed bacteria desolved in distilled water is effective by immersing as well as that of formalin-killed bacteria (Kawano et al, 1984). Furthermore, since efficacy of immersion vaccine using LPS extracted from *V. anguillarum* is also revealed in ayu, LPS is involved in antigen of *Vibrio* immersion vaccine (Aoki et al, 1984).

Glossary

Chr: Chromosome,

ITB: Iron-transport biosynthesis,

PBS: Phosphate buffered saline,

Ig: Immunoglobulin,

PCR: Polymerase chain reaction,

LAMP: Loop-mediated Isothermal Amplification,

ITS: Intergenic spacer region,

OTC: Oxytetracycline,

SA: Sulphonamides,

OA: Oxolinic acid