EXPERIMENTAL INFECTION OF *Vibrio harveyi* IN DIFFERENT WATER SALINITY IN *Epinephelus fuscoguttatus*, TIGER GROUPER

RUZAINI BINTI AHMAD

A DISSERTATION SUBMITTED AS A PARTIAL FULFILLMENT OF THE REQUIREMENTS TO GRADUATE AS A BACHELOR OF SCIENCE WITH HONOURS

AQUACULTURE PROGRAMME
SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITY MALAYSIA SABAH

2010

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS@

JUDUL: Experimental Infection of Vibrio harveyi IN Different Water Salinity In Epinephelus fuscoguttatus, Tiger grouper

Ijazah:

SESI PENGAJIAN: 2009/2010

Saya ~~Rozaini binti Ahmad~~ RUZAINI BINTI AHMAD

(HURUF BESAR)

mengaku membenarkan tesis (LPS/Sarjana/Doktor Falsafah)* ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. **Sila tandakan (/)

[ ]	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)
[ ]	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
[/]	TIDAK TERHAD	

Disahkan oleh: NURULAIN BINTI ISMAIL

LIBRARIAN
UNIVERSITI MALAYSIA SABAH

(TANDATANGAN PENULIS)

(TANDATANGAN PUSTAKAWAN)

Alamat Tetap: Lot Kawi Heights Blok Idaman, D-5-4, Kinarut, Sabah

Dr. Julian Ransangan
Nama Penyelia

Tarikh: 06/05/2010

Tarikh: 06/05/2010

CATATAN: * Potong yang tidak berkenaan.

** Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.

@ Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau secara kerja kursus dan penyelidikan, atau Laporan Projek Sarjana Muda

DECLARATION

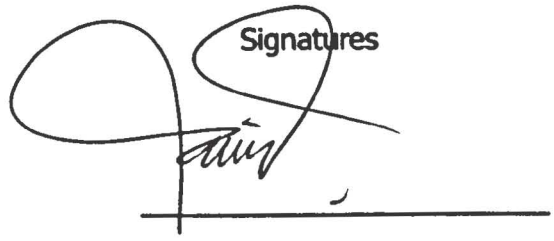
I declare that all contents in this dissertation is the results of my own independent work, except where otherwise stated.

RUZAINI BINTI AHMAD
(BS07110258)

6th APRIL 2010

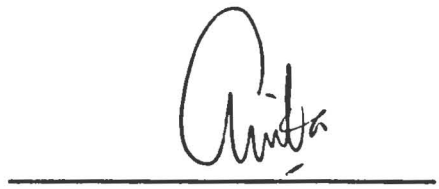
AUTHENTICATION

1. **SUPERVISOR**
(DR.JULIAN RANSANGAN)

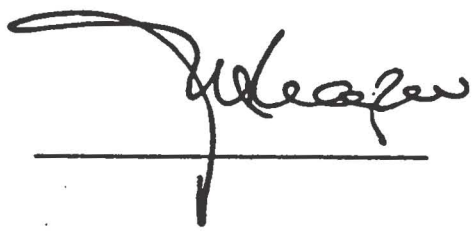


2. **EXAMINER 1**
(DR. ROSSITA SHAPAWI)

3. **EXAMINER 2**
(DR. ANNITA YONG SEOK KIAN)



4. **DEAN**
(PROF. DR. MOHD HARUN ABDULLAH)



ACKNOWLEDGEMENT

First and foremost I want to thank God for giving me the strength and chance to finish this dissertation works.

I would like to express my deepest gratitude to my supervisor, Dr. Julian Ransangan for his guidance, patients and constant support in helping me to conduct and complete my work.

I would also like to thank research assistant of Borneo Marine Research Institute (BMRI) supervised by Dr. Julian who are Mr. Tamrin Lal and Mr. Benny Obrain for their constant encouragement and expertise.

I want to thank Sabah Fisheries Department for their generosity in supplying me *Epinephelus fuscoguttatus* as my experimental fish.

I owe my sincere appreciation to my family and relatives who have supported and encouraged me over the years. Finally, I want to extend my profound appreciation to my beloved parents for their love, affection and invaluable support during my life and studies. I also want to thank all my course mates directly or indirectly for their companionship during my night stay in the laboratory.

ABSTRACT

This experiment was done in order to examine the effect of *V. harveyi* to *E. fuscoguttatus* through experimental infection. *V. harveyi* were inserted in five different water salinities which were 10 ppt, 15ppt, 20 ppt, 25 ppt and 30 ppt. The parameters that examined were mortality rate of the fish species, bacteria colony formation in the organ and bacteria colony formation in the water that inserted with the bacteria. The bacteria colony formations were calculated by incubating a sample of the bacteria from the water and organ thus counting the bacteria colony formed after 12 hours. Besides, the external and internal signs of the infection were also observed. The internal clinical signs were observed by histology process. The pathogenicity of the bacteria determined as the colony formation of the bacteria increased. This experiment shows that there is a significant difference in 30 ppt water salinity in terms of bacterial growth in the water and organ. There was no significant difference in 25 ppt, 20 ppt, 15 ppt and 10 ppt. The bacteria colony formation rate of *V. harveyi* increased with the increasing of water salinity. This experiment shows that 15 ppt gives slowest rate of mortality to the infected fish. Furthermore, the duration of time the fish showing the clinical signs also very slow if compared to the other fish in other salinity. There were no control fish died. The internal clinical signs were observed after histology process shows that *V. harveyi* infection caused lesions, necrosis, hemorrhages, shrunken of the blood vessel and thickening of the muscle while external signs are lethargy, swim spastically, swim abnormally and loss of swimming appetite. It is recommended to culture tiger grouper in 15 ppt.

Keywords: V. harveyi, water salinity, experimental infection, *E. fuscoguttatus.*

ABSTRAK

Eksperimen ini dilakukan bertujuan untuk menguji kesan jangkitan *V. harveyi* kepada *E. fucoguttatus* melalui jangkitan eksperimen. *V. harveyi* dimasukkan ke dalam lima kadar kemasinan air yang berbeza iaitu 30 ppt, 25 ppt, 20 ppt, 15 ppt dan 10 ppt. Parameter yang diuji adalah kadar kematian ikan tersebut, pembentukan koloni bakteria di dalam air dan organ yang telah dimasukkan bakteria tersebut. Proses pengiraan pembentukan koloni bakteria tersebut dilakukan dengan meletakkan bakteria tersebut pada keadaan yang sesuai untuk pertumbuhannya selama 12 jam lalu mengira bilangan koloni yang tumbuh di atas piring petri. Selain itu, tanda-tanda jangkitan luaran dan dalaman juga diperhatikan. Tanda-tanda jangkitan dalaman diperhatikan melalui proses histologi. Kepatogenikan bakteria di kira dengan pertumbuhan bilangan bakteria tersebut. Eksperimen ini menerangkan bahawa terdapat signifikasi perbezaan dari segi pertumbuhan bakteria tersebut di dalam 30 ppt air. Walaubagaimana pun, tidak terdapat perbezaan yang signifikan pertumbuhan bakteria tersebut di dalam 25 ppt, 20 ppt, 15 ppt dan 10 ppt. Eksperimen ini menunjukkan bahawa 15 ppt air memberikan kesan kadar kematian ikan yang paling perlahan. Selain itu, tempoh masa untuk ikan menunjukkan tanda-tanda klinikal juga adalah perlahan. Tiada kematian di dalam ikan control. Tanda-tanda klinikan dalaman yang ditunjukkan adalah lesion, nekrosis, hemorrhage, pengecutan salur darah dan penebalan tisu dinding pada jantung. Manakala tanda-tanda klinikal luaran adalah ikan menjadi kurang aktiv, berenang spastik, berenang secara abnormal dan hilang selera makan. Oleh itu, adalah dicadangkan untuk mengkultur *E. fuscoguttatus* di dalan air 15 ppt.

Kata kunci: *Vibrio harveyi*, kemasinan air, jangkitan melalui eksperimen, *E. fuscoguttatus*

CONTENTS

LIST OF TABLES

LIST OF PHOTOS

LIST OF SYMBOLS, ABBREVIATIONS AND UNITS

BMRI	Borneo Marine Research Institute
cm	centimeter
cfu	colony formation unit
°C	degree centrigrade
D.O	dissolved oxygen
h	hour
µ	micro
ml	milliliter
NaCl	natrium chloride
PBS	phosphate buffer saline
ppt	parts per thousand
%	percentage
pH	potentiometric hydrogen
rpm	revolutions per minute
TSB	Trypticase Soy Broth
TSA	Trypticase Soy Agar
UMS	University Malaysia Sabah

CHAPTER 1

INTRODUCTION

1.1 Aquaculture

Aquaculture or also know as aquatic farming is defined broadly as an intervention in the rearing process that enhances production, such as feeding and protection from predators (Liao *et al.,* 1998). Aquaculture has been tremendously developed during past decades and produce fish products as a main source of protein consumed by human (Amirkolaie *et al.,* 2008).

This industry is the fastest growing food-producing sector in the world if compared with terrestrial animals. Asia has contributed 90% of the world aquaculture production (Zilong *et al.,* 2004). This industry involves cultivating aquatic populations under controlled conditions and can be contrasted with commercial fishing which is the harvesting of wild fish. Commercial aquaculture supplies one half of the fish and shellfish that is directly consumed by human (Cano-Gomez *et al.,* 2007).

Aquaculture in Asia has a long history of more than 2,500 years and is recognized as the leading aquaculture region in the world, contributing to 90% of total world aquaculture production. There are over a hundred species of finfish cultured in the region. Marine aquaculture in Asia has been developed rapidly and is the fastest growing sub-sector in Asia. Much of this increasing production is attributable to the expanding culture of high value marine species such as groupers (Angulo, 2000).

1.2 Constraints in Aquaculture

Aquaculture industry is very profitable. However, from the time man started to culture fish, fish disease has changed from being an interesting phenomenon to and important socio-economic problem. Infectious disease is considered to be the industry's single most important cause of mass mortalities and economic losses. Health problem has two fiscal consequences on the aquaculture industry which are loss of productivity due to animal mortality and morbidity, and loss of trade due to food safety issues as reported by Arthur (1995).

Disease is undoubtedly recognized as one of the biggest constraints to the production, development and sustainable expansion of aquaculture in the Asian region. Most farms operate on a small scale and with limited technical support, disease diagnosis and training is usually lacking at the farm level. Even if fish suffer from disease and overall survival is low, epidemiology data are rarely collected, reported and analyzed (Bondad- Reantaso *et al.,* 2005).

In the past few years, more attention has been given to the identification of etiological agents involved in fish disease epidemics. Pathogens can be classified to either parasitic, viral, bacterial and fungal groups.

Due to lack of diagnosis, farmers often apply antibiotic treatment when mortality rises without knowing the cause of the disease and assuming that it is caused by a bacterial pathogen. Some farmers even use antibiotics as a form of 'preventative measure' where antibiotics are administered in the anticipation of an expected disease outbreak. This has resulted in the use of chemicals and drugs (Choo *et al.,* 2000). While under certain circumstances antibiotics can provide a useful means of reducing the adverse effects of bacterial diseases. An important side effect of the use of antibacterial drugs in aquaculture is the development of drug resistance among the fish and shellfish bacterial pathogens (MacMillan *et al.,* 2001).

Good farming and health management practices are still to be implemented. For example, the use of trash fish as feed is a common practice in small scale marine fish farming. From a health management perspective, the use of trash fish as feed opens the door to a variety of potential pathogens and infections. Furthermore, it is one of the major causes of fish diseases in Asia.

Pathogenic diseases in fish have become a matter of concern in aquaculture industry. *V. harveyi* has been widely recognized as a primary pathogen of many commercially cultured fish in the whole world and an economically significant pathogen for aquaculture marine fish. It has been associated with mass mortality events in other aquaculture species such as groupers, prawn, shrimp and mollusk as (Bourne *et al.*, 2004). Due to this reason, it is important to study the environmental factor of the growth and its pathogenicity to control and thus minimizing the chance for the bacteria to evolve. According to Alavandi (2006), *V. harveyi* growth rate varies at different salinity. For instance, by adjusting and experimenting the ideal water salinity, the growth rate of the bacteria can be limited. Thus, the usage of antibiotics and chemicals can be reduced. Besides, the environmental stress can be loosen.

By only assuming the cause of death without proper reference does not improve the rearing technique. This is because not all aquatic disease needs to be cured with antibiotics or chemicals. There are lots more techniques that can prevent and solve the problems on aquatic diseases. Environmental conditions often become the factor of disease outbreak such as different water salinity, pH and dissolved oxygen (Ramesh *et al.*, 1989). This is because *V. harveyi* and other bacteria, parasites and virus have their specific adaptability condition. For instance, we can rear the aquatic animal such as fish at the unadaptability condition or minimal adaptability condition of the bacteria to stop or minimize the growth rate.

1.3 Study Objective

The major objective of this experiment is to examine the pathogenicity of *Vibrio harveyi* in difference water salinity to *Epinephelus fuscoguttatus*, tiger grouper.

CHAPTER 2

LITERATURE REVIEW

2.1 Experimental Bacteria, *Vibrio harveyi.*

V. harveyi mainly found in tropical waters. This is one of the bacteria species that has caused a lot of economic losses in aquaculture industry (Kakizaki *et al.,* 2009). Understanding the environmental living condition of this species especially in the different water salinity may help in minimizing the infection rate.

V. harveyi is in the phylum of Proteobacteria, class of Gammaproteobacteria and the order of Vibrionales. It is in the family of Vibrionaceae. *V. harveyi* also known as *Lucibacterium harveyi, Beneckea harveyi, Achromobacter harveyi, Pseudomonas harveyi, Vibrio carchariae* and *Vibrio trachuri* (Thompson *et al.,* 2004).

V. harveyi is a pathogen of fish and invertebrates including groupers, seabass, sharks, lobsters and shrimp. Its pathogenicity depends on the concentration of *Vibrio harveyi* cells at given times. Diseases caused by this species include eye lesions, gastro-enteritis, vasculitis and luminous vibriosis (Austin *et al.,* 2006). Luminous vibriosis is the leading cause of death among commercially farmed groupers, shrimp and other aquaculture species. The infection by this species enters through the mouth and forms plaque, then spreads to the inards and appendages. Loss of limb functions and appendage degradation occur. Contamination can spread all the way to egg and larval tanks, thus causing big problem to the fish farmer (Lightner *et al.,* 1993).

The living optimal temperature of the bacteria is between 28°C to 30°C. It is a gram-negative bacteria that is rod in shape. It can live in aerobic and anaerobic condition. The species has flagella that allowed it to move. Furthermore, it does not form symbiotic relationship unlike its close relative which is *V. fischeri* (Austin *et al.*, 2006).

Most luminous bacterial disease including *V. harveyi* outbreaks occur during rainy season (Sunaryanto *et al.*, 1986). This indicates that environmental factors such as temperature, salinity, pH and organic loading involve in triggering disease outbreaks (Ramesh *et al.*, 1989).

2.2 Experimental Species *Epinephelus fuscoguttatus*, Tiger Grouper

Epinephelus fuscoguttatus also known as tiger grouper, brown-marbled grouper and flowery cod. This is a species of Serranidae family. According to Charnov (1982) this species is a protogynous hermaphrodite which means that this fish first function sexually as females and later change its sex to male as the age increase. It is also one of the largest fish predators on coral reefs and is mainly active at dusk, when it feeds on fishes, crabs and cephalopods.

Tiger grouper is among the highest value fish in the trade and consequently favored by the farmers (Sadovy *et al.*, 2003). This species is favored by the farmers for its hardiness and its rapid growth if compared to Humpback grouper as reported by Labrie (2005).

The species most distributed in tropical marine waters and also found in freshwater. It can be found in lagoon pinnacles, channels, outer reef slopes and in coral-rich areas with clear waters. The juveniles mostly found in seagrass beds (Kohno et al., 1993).

Hatchery production of brown-marbled juveniles is still below demand and is constrained by poor and unreliable survival of larvae in hatcheries. The supply of this

Hatchery production of brown-marbled juveniles is still below demand and is constrained by poor and unreliable survival of larvae in hatcheries. The supply of this species has declined in many areas that involved overfishing of the adults and seed, habitat destruction, pollution, high export demand and disease infection (Hellio *et al.*, 2002). Vibriosis caused by *V. harveyi* is one of the major bacteria that contribute to the mortality of brown-marbled grouper juvenile in hatchery but there is still not much study on the infections of *V. harveyi* in tiger grouper.

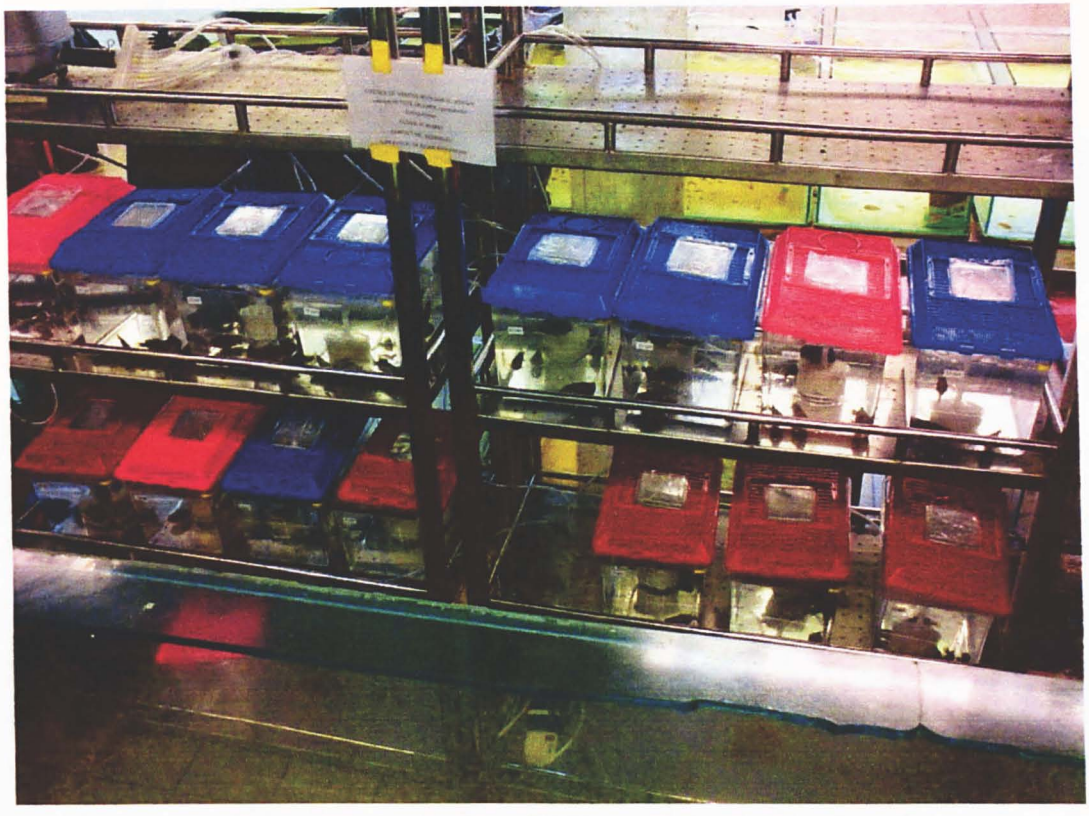


Photo 3.1 Experimental aquaria arrangement in UMS wet laboratory.

Aquaria were filled with varying salinities which ranging from 10 ppt, 15 ppt, 20 ppt, 25 ppt and 30 ppt. Furthermore, the water quality were maintained everyday by checking the water quality parameter which are dissolved oxygen (D.O), pH and temperature. The temperature was ranging between 28 to 30°C while pH was between 6.8 to 7.3. D.O were maintained between 6.7 mg/l to 7.7 mg/l. In order to sustain the water quality, bottom cleaning were done daily after feeding the fish. Photo 3.2 shows the random arrangement of the aquaria. Each treatment immersed with V. *harveyi* except the control aquaria. C symbol in the photo indicate the control aquaria.

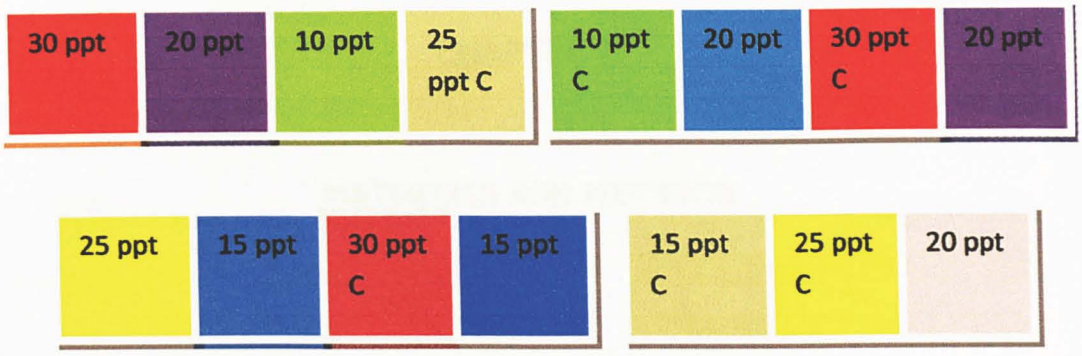


Photo 3.2 Random arrangement of the aquaria.

3.2 Media Preparation

The media used in this experiment were Trypticase Soy Agar (TSA), Trypticase Soy Broth (TSB), Phosphate Buffer Saline (PBS) and Vibrio Agar. Trypticase Soy Agar is an enriched medium containing 1.5% trypticase peptone, 0.5% phytone peptones, 0.5% NaCl, 1.5 % agar that supports the growth of many fastidious organisms like *Streptococci sp.* and some members of the genera *Neisseria, Brucella, Vibrio and Pasteurella* (Cunnif *et al.,* 1993). While Trypticase Soy Broth is used for the cultivation of a wide variety of microorganisms including vibrio genera in the form of liquid with the addition of deionized water. In the preparation of TSB media, 9 grams of TSB powder added with 4.5 grams of NaCl and 300 ml ionized water. While TSA prepared by the mixing of 16 grams of TSA powder with the addition of 6 grams of NaCl and 400 ml of deionized water. Vibrio agar prepared with the mixture of 32 grams of vibrio agar powder and 400 ml of deionized water. PBS was prepared in four 500 ml bottle with the amount of 400 ml each. TSA, TSB and Vibrio agar that already been mixed were boiled in a water bath. TSA and Vibrio agar were then poured into disposable petri dish until they totally turn into agar solid form. While TSB which is in the form of liquid transferred into 2 types of bottle which were 500 ml bottle and 5 ml bottle. TSA, 5 ml TSB and the Vibrio agar kept in the refrigerator before being used. Photo 3.3 shows

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Design

E. fuscoguttatus juvenile were taken from Tanjung Badak Fisheries Department, Tuaran, Sabah were stocked in a tank to assess their disease free health status through observation of clinical signs for a period 15 days. The healthy fish were acclimatized in ambient laboratory condition and fed with trash fish.

The experiment was run at Borneo Marine Research Institute (BMRI) University Malaysia Sabah (UMS) wet laboratory. Photo 3.1 shows the aquaria arrangement along the experiment. Photo 3.2 showed rectangular aquarium with the capacity of seven litres were stocked with five tails of *E. fuscoguttatus* juvenile with the average total length of 8.5 cm. There are 15 aquaria which consist of five duplicate treatments and five control. The aquaria were arranged randomly and the experiment duration was 10 days.

3.4 Bacteria Challenge

The bacteria suspension was seeded at 15 ml into the water in each treatment aquarium except in the control aquaria. Duration of the challenge was 10 days. The tiger grouper observed continuously. Death and clinical signs were recorded along the challenge. Bacteria counting were done daily by collecting 1 ml of water sample from each aquaria.

3.5 Bacteria Counting

Dead fish was dissected for bacterial counting in the kidney and liver. The organs were crushed into smaller pieces. Then, 0.1ml of the crushed organs transferred into seven tubes of the microcentifuge tube containing 900 µl of PBS. At first, the 0.1 ml crushed organ transferred to the first microcentrifuge tube containing 900 µl of PBS and shook. Then, 0.1 ml of the first mixture transferred into the second microcentrifuge tube and again, shook. This step repeated until the seventh mirocentrifuge tube to prepare a seven time serial dilution mixture. The colony formation were calculated with the formula below.

Amount of bacteria (cfu/ml) = (Amount of bacteria counted on the plate) x 15ml x 10^7

There are three parts where bacteria counting is vital. It is important to measure the initial amount of *V. harveyi* before immersed, the growth rate of bacteria immersed in the water and the final amount of the bacteria in the organ that caused the fish to die.

3.5.1 Initial Amount of *Vibrio harveyi* Before Insertion

After the bacteria that cultured in 12 hours taken out, 0.1 ml of it pipeted and serial dilution was done. The initial amount of *V. harveyi* was counted by seven times serial dilution. After that, 0.1 ml of the seventh dilution spread onto the Vibrio agar. In order

to avoid bacteria contamination, the spreading rod glass was immersed in a 90% ethanol concentration and sterile by heating it with a Bunsen's burner. After that, the bacteria incubated at 28°C for 12 hours. After 12 hours, the incubated bacteria counted and the amount recorded.

3.5.2 Colony Formation of Bacteria in the Water Inserted

The water in each aquaria that inserted with *V. harveyi* were collected daily. The water was autoclaved and different tips were used in each water salinity to avoid contamination of bacteria from other tank. It is also counted by serial dilution method at seven times dilution. The bacteria incubated at 28°C for 12 hours (Baumann *et al.,* 1984). The amount of colony calculated and recorded. The bacteria colony formation rate of *V. harveyi* in the water calculated using the formula below.

Bacteria colony formation rate of *V. harveyi* in the water=

$$\frac{\text{Sum of all the bacteria counted (cfu/ml)}}{\text{Days after bacteria immersion}}$$

(Sunaryanto *et al.,* 1990)

3.5.3 Amount of the Bacteria in the Organ

Dead fish was dissected for kidney and liver samples. The organ crushed into smaller pieces. Then, 0.1 ml of the crushed organs transferred into seven tubes of the microcentifuge tube containing 0.9 ml of PBS. At first, the 0.1 ml crushed organ transferred to the first microcentrifuge tube containing 0.9 ml of PBS and shook. Then, 0.1 ml of the first mixture transferred into the second microcentrifuge tube and again, shook. This step repeated until the seventh tube. The mixture were pipeted at 0.1 ml then spread onto the Vibrio agar. After incubated for 12 hours, the bacteria colony formed counted and recorded. The colony formation rate of *V. harveyi* in the organ was calculated using the formula given.

REFERENCES

Agius, C. 1985. The melano-macrophage centres in fish: a review. In: Manning, M.J., Tatner, M.F. (eds.), Fish Immunology. *Academic Press, London*, 85-105pp.

Alavandi, S.V., Manoranjita, V., Vijayan, K.K., Kalaimani, N., Santiago, T.C. 2001. Phenotypic and Molecular Typing *Vibrio harveyi* isolates and their pathogenicity to tiger shrimp larvae. *Letters in Applied Microbiology*, **43**: 566-570.

Amaro, C., Biosca, E. G.,Esteve, C., Fouz, B., Toranzo, A. 1992. Comparative Study of Phenotypic and Virulence Properties in *Vibrio harveyi* biotype 1 and 2 obtained from a European eel farm experiencing mortalities. *Diseases of Aquatic Organisms*, **13**: 29-35.

Amirkolaie, A., Bai, S.C., Kim K.W., 2008. Present status and future prospects of Aquaculture in Korea. *World Aquaculture*, **32**: 28-32.

Angulo, F. 2000. Antimicrobial agents in aquaculture: potential impact of public health. *Apua Newsletters*, **18**: 1-4.

Arthur, J.R., Ogawa, K. 1996. A brief overview of disease problems in the culture of Aquaculture Health Management Strategies for Marine Fishes. *Proceedings a workshop in Honolulu, Hawaii,* October 9-13, 1995, The Oceanic Institute, Hawaii, 9-31pp.

Austin, B., Zhang, X. H. 2006. *Vibrio harveyi:* a significant pathogen of marine invertebrates. China. *Letters in Applied Microbiology,* **43**: 199-124.

Balebona, M.C., Morinigo, M.C., Faris, A., Krovacek, K., Mansson, I., Bordas, M.A., Borrego, J.J. 1999. Influence of Salinity and pH on the Adhesion of Pathogenic *Vibrio* Strains to *Sparus aurata,* Skin Mucus. 1999. Department of Microbiology, Faculty of Sciences, University of Malaga, Sweden.

Bartlett, M.S. 2004. *Methods of PCR in Molecular Biology.* Institute de Biologie Moleculaire des, France. 183-192pp.

Bautista, M. N., Lavilla- Pitogo, C.R., Subosa, P.F. 1994. Histopathology of the hepatopancreas and gills of the prawn *Macrobrachium malcolmsonii* exposed to vibriosis. *Aquatic toxicology,* **50** (2000): 330-339.

Beachy, E.H. 1981. Bacterial adherence: Adhesion receptor interactions mediating the attachment of bacteria to mucosal surfaces. *Journal of Bacterial Infection in Fish,* **143**: 325-345.

Bondad-Reantaso, M., Subasinghe, R.P., Arthur, J.R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z., Shariff, M. 2005. Disease and Health Management in Asian Aquaculture. *Parasitology,* **132**: 249-272.

Bourne, D.G., Young, N., Webster, N., Payne, M., Salmon, M., Demel, S., Hall, M. 2004. Microbial community dynamics in a larval aquaculture system of the tropical rock lobster, *Panulirus ornatus. Aquaculture,* **242**: 31-51.

Cano-Gomez, A., Bourne, D.G., Hall. M.R., Owens, L., Hoj. L. 2007. Molecular identification, typing and tracking of *Vibrio harveyi* in aquaculture systems: Current methods and future prospects. PhD thesis. *Australian Institute of Marine Science,* 76pp.

Charnov, E.L. 1982. The Theory of Sex Allocation. *Princeton University Press. Princeton.*

Choo, P.S. 2000. Antibiotic Use in Aquaculture: The Malaysian Perspective. *Infofish International,* **2**: 24-29.

Csaikl, U.M., Bastian, H., Brettschneider, R., Gausch, S., Meir, A., Schauerte, M., Sperisen, C., Vornam, B., Ziegenhagen, B. 2004. Comparative Analysis of Different DNA Extraction Protocols: A Fast, Universal Maxi- Preparation of High Quality Fish DNA. *Fish Molecular Biology*, **16**: 69-86.

Ellis, A.E. 1980. Antigen-trapping in the spleen and kidney of the plaice, *Pleuronectes platessa*. *Letters of Applied Microbiology*, **3**: 413-426.

Fouz, B., Devesa, S., Gravningen, K., Barja, J. L., Toranzo, A.E. 1990. Antibacterial action of the mucus of turbot. *European Association of Fish Pathology* , **10**: 56-59.

Garcia, E., Mi W.K., Park, S. 1997. Pathogenicity of *Vibrio harveyi* to cultured marine fishes in Korea. *Aquaculture*, **285**: 1-4.

Hellio, C., Pons, A. M., Beaupoil, C., Bourgougnon, N., Le Gal, Y. 2002. Antibacterial, Antifungal and Cytotoxic Activities of Extracts from Fish Epidermis and Epidermal Mucus. International Research Antimicrobial Agents, **20**: 214-219.

Kohno, H., Diani S., Supriatna A. 1993. Morphological development of Larval and Juvenile Grouper, *Epinephelus fuscoguttatus*. *Japan Journal of Ichthyology*, **40** (3): 307-316.

Jennings, S., Reynolds, J.D. 1999. Predicting the Vulnerability of Tropical Reef Fishes to Exploitation with Phylogenies and Life Histories. *Conservative Biology*, **13**: 1466-1475.

Kakizaki, E., Kozawa, S., Sakai, M., Yukawa, N. 2009. Bioluminescent bacteria have potential as a marker of drowning in seawater: Two immersed cadavers retrieved near estuaries, Tokyo. *Journal of Legal Medicine*, **11**: 91-96.

Karunasagar, I., Pai, R., Malathi, G.R. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture*, **128**: 203-209.

Kiriratnikom, S., Ruangsri, J., Wanadet, M., Songpradit, A., Suanyuk, N., Supamattya, W. 2000. The abiotic factors influencing the growth of luminescence bacteria, *Vibrio harveyi* in seawater. *Songklanakarin Journal of Science and Technology*, **22**: 697-705.

Kraxberger-Beatty, T., Mc Gary, D.J. 1990. *Vibrio harveyi*, an opportunistic pathogen of common snook, *Centropomus undecimalis* (Bloch), held in captivity. *Journal of Fish*, **13**: 557-560.

Labrie, L., Ng, Z. T., Komar, E. C., Grisez, L. 2005. Nocardinal infection in fish: an emerging problem in freshwater and marine aquaculture systems in Asia. In: Bondad-Reantaso, M., (eds) *Diseases in Asian Aquaculture*. Fish Health Section, Asian Fisheries Society, Combo, Sri Lanka.

Lavilla-Pitogo, C.R., Baticados, M.C.L., Cruz-Lacierda, E.R., De la Pena., L.D. 1990. Occurance of the Luminous Bacterial Disease of *Penaeus monodon* larvae in the Philipines. *Aquaculture*, **91**: 1-13.

Liao, I.C. 2000. *The State of Finfish Diversification in Asian Aquaculture.* Zaragoza International Fisheries Institute.

Lightner, D. V. 1993. Diseases of cultured penaeid shrimp. *Journal of Crustacean Aquaculture*. 393–486pp.

Lin, J., Ana, C. G., David, Bourne D. G., Leigh, O. 2009. Molecular identification, typing and tracking of *Vibrio harveyi* in aquaculture systems: Current methods and future prospects. *Aquaculture*, **287**: 1-10

Mac Millan, J.R. 2001. Aquaculture and Antibiotic Resistance: A negligible public health risk. *Journal of World Aquaculture*, **12**(14): 49-68.

Pedersen, K., Verdonck, L., Austin, B., Austin, D.A., Blanch, A.R., Grimont, P.A.D., Jofre, J., Koblavi, S., Larsen, J.L., Tianinen, T., Vigneulle, M., Swings, J. 1998. Taxonomic evidence that *Vibrio carchariae* is a junior synonym of *Vibrio harveyi*. *International Journal of Sysyematic Bacterology*, **48**:749-758.

Prayitno, S.B., Latchford. 1995. Experimental infections of crustaceans with luminous bacteria related to Photobacterium and Vibrio *sp.* Effect of salinity and pH on infectiosity. Department of Fisheries, Faculty of Animal Science, Indonesia.

Press, C.M. 1999. The morphology of the immune system in teleost fishes. *Fish and Shellfish Immunology*, **9**:309-318.

Ramesh, A., Loganathan B.G., Venugopalan V.K. 1989. Seasonal distribution of luminous bacteria in sediments of tropical estuaries. *Journal of Genetic Application of Microbiology*, **35** (5): 363-368.

Rasheed, O. H., Ito, H., Ishigaki, I. 1992. Distribution of pathogenic vibrios and other pathogenic vibrios and other bacteria in imported frozen groupers and their decontamination by gamma-irradiation. *World Journal of Microbiology and Biotechnology*, **8** (5): 494-499

Reichelt, J.L., Baumann, L. 1973. Taxonomy of the marine luminous bacteria. *Archaeology of Microbiology*, **94**: 283-330.

Sadovy, Y.J., Donaldson, T.J., Graham, T.R., McGilvary, F. 2003. *While Stocks Last: The Live Reef Food Fish Trade*. Asian Development Bank, Manila, Phillipines.

Saeed, M.O. 1994. Efforts to control outbreaks of diseases among cultured fish in Kuwait. In: *Proceeding Symposium of Technology and Investment Opportunities*, 11-14 April 1993, Riyadh, Saudi Arabia. 402-411pp.

Secombes, C.J., Fletcher, T.C., White, A., Costello, M., Stagg, R., Houlihan, D. 1992. Effects of sewage sludge on immune responses in the dab. Aquatic Toxicology, 23: 217.

Sunaryanto, A., Maniam, A. 1986. Occurance of Pathogenic Bacteria Causing Luminescence in Penaid Larvae in Indonesian Hatcheries, Brackish Aquaculture Development Centre. *Journal of Pathogenic Bacteria,* **8** (2): 64-70.

Supriyadi, H., Rukyani, A. 1992. The use of antibiotics and drugs for the treatment of bacterial disease of fish and shrimp in Indonesia. In: Shariff, M., Subasinghe, K., Julian, A (eds) Disease in Asian Aquaculture 1. *Proceedings of the First Symposium on Disease in Asian Aquaculture, Bali, Indonesia.* Fish Health Section, Asian Fisheries Society, Philiphines. 515-518pp.

Tan, Z., Grisez, L. 2004. Health Management Practices in Asian Mariculture: Current Status and Challenges. *Proceeding of the 7th Asian Fisheries Forum*, November 2004, Asian Fisheries Society. Penang, Malaysia.

Thompson, F.L., Iida, T., Swings, J. 2004. Biodiversity of vibrios. Microbiology, Mol. Biology Rev. **68**: 403-431.

Won, K. M., Choi, J.H., Park, S. I., 2006a. Characterization of the extracellular products (ECPs) of *Vibrio harveyi* grown under various conditions. *Journal of Fish Pathology,* **19**:119-126.

Won, K.M., Kim, S. M., Park, S.I., 2006b. Characterization of *Vibrio harveyi,* the causal agent of vibriosis in cultured marine fishes in Korea. *Fisheries of Science and Technology,* **9**:123-128.

Zilong, T., Cedric, K., Enright, W.J. 2003. *Health Management Practices For Cage Aquaculture in Asia: A Key Component For Sustainability.* Intervet International Fisheries, Singapore.