UNIVERSITI PUTRA MALAYSIA

EFFECTS OF QUORUM SENSING AND ITS DEGRADER ON THE VIRULENCE OF Vibrio harveyi TOWARDS TIGER GROUPER, Epinephelus fuscoguttatus Forsskål LARVAE

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Quorum sensing (QS) is a bacterial cell-to-cell communication with small signal molecules such as acyl-homoserine lactones (AHL) that control a number of phenotypes including the regulation of virulence determinants in pathogenic bacteria. Therefore, quorum sensing degrader has been suggested as one of the biocontrol strategy to fight bacterial infections as an alternative to the use of antibiotics. In this study, the link between quorum sensing (QS) in *Vibrio harveyi* and its virulence towards tiger grouper (*Epinephelus fuscoguttatus*) were investigated. The virulence activity was studied using *V. harveyi* wild type and its QS mutants with constitutively maximal or minimal quorum sensing activity and different signal molecule (autoinducer) synthase gene mutants. The potential of live feed *Artemia* as the carrier of bacterial quorum sensing degrader for the tiger grouper larvae was also investigated. The results showed that the wild type *Vibrio harveyi* BB120 was pathogenic to grouper (*Epinephelus fuscogutattus*) larvae causing more than 50% larval mortality after four days of challenge. Furthermore, the mortality of grouper larvae challenged with*V. harveyi*mutant JAF483 with maximally active QS (QS+) was significantly higher than the *V. harveyi*wild type BB120. Meanwhile, high survival was observed in the grouper larvae challenged to JAF548 with minimally active QS (QS-) compared to the JAF483 (QS+). High survival of larvae were also observed in the QS autoinducer mutant strains of JMH634 (QS triple autoinducer synthase mutant), BB152 (Harveyi autoinducer-1 (HAI-1) synthase mutant) and MM30 (Autoinducer-2 (AI-2) synthase mutant). In contrast, low survival was observed in the strain JMH603 (Cholerae autoinducer-1 (CAI-1) synthase mutant). This indicated that the HAI-1 and AI-2 QS signal molecules might play important roles for the virulence of *V. harveyi* towards grouper larvae but not CAI-1. To support this, addition of HAI-1 in the water restored the virulence of the mutant. The effects of the infection on the larvae could also be seen histologically. In addition, the *in vivo* expression data showed that the HAI-1 signal molecule upregulated the innate immune genes consisting of tripartite motif-containing protein 39 (TRIM39), Hepcidin-1 (Hep-1), peptidoglycan recognition protein SC2 (PGRP) and toll like receptor 5 (TLR5) at different hours compared to control. The expression of both control and the bacterial mutant BB152 without HAI-molecules remained low throughout the experimental period. Next, different bacterial QS degrader strains were isolated from *Artemia* and screened using *Chromobacterium violaceum* CV026

bioassay. The results showed six bacterial strains (four Gram-positive and two Gram-negative) isolated from *Artemia* are able to degrade AHL in two different *in vitro* assays. The highest bacterial AHL degrader, identified as *Bacillus litoralis* BP-ART/6 fully degraded 10 ppm AHL from 9 hours. Encapsulation of the *Bacillus* strain to *Artemia* also significantly improve the survival and total length of the live feedcompared to control without addition of any bacteria. This study showed that bacterial strain isolated from *Artemia* can act as a quorum sensing degrader and can be encapsulated to *Artemia*. It was later observed that *B. litoralis* could also be used as probiotic bacteria administered through *Artemia* nauplii to protect grouper larvae against infection of *V. harveyi.*

Keywords: Tiger grouper; *Vibrio harveyi*; Quorum sensing; Quorum sensing degrader.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

KESAN PENDERIAAN KUORUM DAN PERENCATNYA TERHADAP KEVIRULENAN *Vibrio harveyi* KEPADA LARVA KERAPU HARIMAU, *Epinephelus fuscoguttatus* Forsskål

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Penderiaan kuorum (QS) adalah komunikasi bakteria antara bakteria sel ke sel dengan menggunakan molekul isyarat kecil dikenali sebagai '*acylated homoserine laktons*' (AHL) yang mengawal beberapa fenotip termasuk pengaturan virulensi dalam bakteria patogenik. Oleh itu, perencatnya telah dicadangkan sebagai salah satu strategi untuk melawan jangkitan bakteria sebagai alternatif kepada antibiotik.Dalam kajian ini, hubungan antara penderiaan kuorum (QS) di antara *Vibrio harveyi* dan kevirulenannya terhadap kerapu harimau (*Epinephelus fuscoguttatus*) telah disiasat. Aktiviti ini dikaji menggunakan *V. harveyi* jenis liar dan mutan QS dengan aktiviti penderiaan kuorum maksimum atau minimum serta mutan gen bagi sintesis molekul isyarat yang berbeza. Potensi *Artemia* (makanan hidup ikan) sebagai pembawa bakteria perencat QS bagi larva kerapu harimau turut disiasat. Hasil kajian menunjukkan bahawa mutan liar *Vibrio harveyi* BB120 adalah patogenik kepada larva kerapu (*Epinephelus fuscogutattus*) dengan lebih daripada 50 % kematian selepas empat hari. Tambahan pula, kadar kematian larva kerapu dicabar mutan JAF483 dengan aktiviti QS maksima (QS+) adalah jauh lebih tinggi daripada jenis liar BB120. Selain itu, hasil kajian menunjukkan kadar hidup larva kerapu tinggi apabila dicabar dengan JAF548 dengan aktiviti QS minimum (QS-) berbanding dengan JAF483 (QS+). Hasil kajian juga menunjukkan kadar hidup larva yang tinggi dalam JMH634 (mutan QS *triple autoinducer synthase*) , BB152 (mutan *Harveyi autoinducer-1* (HAI-1) *synthase*) dan MM30 (mutan *Autoinducer-2* (AI-2) *synthase*). Sebaliknya, kadar hidup rendah diperhatikan dalam mutan JMH603 (mutan *Cholerae autoinducer-1* (CAI-1) *synthase*). Ini menunjukkan bahawa molekul isyarat QS HAI-1 dan AI-2 mungkin memainkan peranan penting bagi kevirulenan *V. harveyi* terhadap larva kerapu tetapi tidak CAI-1. Untuk menyokong pendapat ini, penambahan HAI-1 di dalam air didapati memulihkan kevirulenan mutan. Kesan jangkitan juga boleh dilihat secara histologi. Molekul isyarat HAI-1 juga meningkatkan gen sistem imun *tripartite motif-containing protein 39* (TRIM39), *Hepcidin 1* (Hep-1), *peptidoglycan recognition protein SC2* (PGRP) dan *toll like receptor 5* (TLR5) pada waktu yang berbeza. Manakala, ekspresi gen imun adalah kekal rendah sepanjang tempoh eksperimen dalam rawatan terkawal dan mutan BB152 tanpa molekul HAI-1. Seterusnya, perencat QS telah diasingkan daripada *Artemia* menggunakan bioassai *Chromobacterium violaceum* CV026. Hasil kajian menunjukkan enam jenis bakteria (empat Gram-positif dan dua Gram-negatif) telah diasingkan daripada *Artemia* dan dapat merendahkan AHL dalam dua asai berbeza. Bakteria perencat QS yang terbaik dikenal pasti sebagai *Bacillus litoralis* (BP/Art-6), mampu merendahkan 10 ppm AHL sepenuhnya dalam 9 jam. Dalam

kajian ini, peningkatan yang ketara dalam hidup dan pertumbuhan dalam *Artemia* dicapai apabila dikultur bersama *B. litoralis*. Ia juga diperhatikan bahawa *B. litoralis* boleh digunakan sebagai bakteria probiotik melalui makanan hidup ikan *Artemia* yang mampu melindungi larva kerapu daripada jangkitan *V. harveyi*.

Kata Kunci: Kerapu harimau, *Vibrio harveyi*, penderiaan kuorum, perencat penderiaan kuorum

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of (Master of Science). The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AHL	Acyl homoserine lactone
AI-2	Autoinducer-2
ATCC	American Type Culture Collection
CAI-1	Chlolerae autoinducer-1
cDNA	complementary DNA
cfu	colony forming unit
DNA	Deoxyribonucleic acid
DO	dissolved oxygen
dpi	days post infection
FAO	Food and Agriculture Organization
H&E	Haematoxylin and Eosin
HAI-1	Harveyi autoinducer-1
HHL	Hexanoyl homoserine lactone
LB	Luria Bertani
MA	Marine agar
MB	Marine broth
MC	microbial communities
mRNA	Messenger RNA
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
OD	optical density
PCR	Polymerase chain reaction
ppm	parts per million
ppt	parts per thousand
QS	Quorum sensing
QSI	Quorum sensing inhibitor
RT-qPCR	Real time quantitative polymerase chain reaction
rpm	rotation per minute
rRNA	Ribosomal ribonucleic acid
SD	standard deviation
TCBS	Thiosulphate citrate bile salt sucrose
µg	microgram
µM	micromolar

CHAPTER 1

INTRODUCTION

Aquaculture is the farming of aquatic animals and plants (Costa-Pierce *et al.,* 2006). It involved the farming of freshwater and saltwater animal species including fishes, molluscs, crustaceans and aquatic plants. Unlike fishing where men remove aquatic organisms out of its habitat for consumption, aquaculture implies the cultivation of aquatic populations under controlled or semi-controlled conditions. Aquaculture has been growing steadily as a feedback to the declining of the world's fisheries due to overfishing. The aquaculture industry continues to expand, diversify and advance technologically dominating other animal-producing sectors in terms of growth (FAO, 2012).

Among the target species under the aquaculture sector are groupers. Currently groupers are cultured in large scales especially in Asian countries due to the advantages of this fish as efficient feed converters, fast growth and good flesh quality. The fish is much sought after by local and international markets, particularly in Asia such as Hong Kong, Singapore and Japan with high market price (FAO, 2012; Ottolenghi *et al.,* 2004). Compared to other grouper species, tiger grouper *(Epinephelus fuscoguttatus)* have high adaptability in captivity (Gunben *et al.,* 2014). However, one of the constraint in grouper production is that the supply of the juvenile fish is limited due to inadequate seed productions. In the hatchery, larval mortality of *Epinephelus fuscoguttatus* at the first feeding was common (Liao *et al.,* 2005). High densities of larvae and organic matter (live feed, faeces or from dead larvae debris) lead to the development of pathogens e.g., viruses, parasites and pathogenic bacteria causing poor growth or mass mortality. The control of pathogens in the live feed production and rearing systems are among the determinant aspects for the larvae endurance (Dhert *et al.,* 2001; Shirakashi *et al.,* 2013).

Of all pathogens, vibriosisis among the common bacterial disease which affect various grouper culture stages (Manin and Ransangan, 2011). *Vibrio harveyi* are among the important bacterial pathogen in finfishes (Pietrak *et al.,* 2012), molluscs (Romalde *et al.,* 2013) and shrimps (Manilal *et al.,* 2010) culture which result in crucial financial losses in the farms and hatcheries (Laffert*yet al.,* 2015). Mainly, the ways for the control of bacterial infection is either to inhibit the growth of the microorganism or to attenuate its virulence leading to the failure of the organism. In aquaculture, antibiotics have been used to treat vibriosis. However, several pathogenic strains have shown resistance towards antibiotics. In juvenile fish, vaccination is given to treat diseases and it has lowered the use of chemotherapeutics in aquaculture and increased the survival of cultured fish. However, Vadstein (1997) stated vaccines are not suitable for larvae since the organisms are small and does not have a developed immune system and mainly depend on the non specific immune response and maternal antibodies. Thus, several researches continue to find new approach to a more environmentally friendly and sustainable control of vibriosis in the larvae culture systems as an alternative to antibiotics and vaccination.

The discoveries that bacteria use quorum sensing molecules, to regulate the production of virulence determinants and secondary metabolites could be a novel target to overcome bacterial diseases (Hamza *et al.*, 2015). In quorum sensing, bacteria communicate with one another by releasing, detecting and responding to the small signal molecules, also known as autoinducers. Detection of autoinducers allow bacteria to distinguish between low and high cell population density and regulate gene expression in response to changes in cell numbers. Many bacterial behaviours such as symbiosis, virulence, antibiotic production and biofilm are regulated by quorum sensing (Schauder and Bassler, 2001). It is shown that highly specific as well as universal quorum sensing languages exist, which enabled bacteria to communicate within and between species.

Interference of quorum sensing has been suggested as a new anti-infective strategy in bacteria. Several methods has been proposed to interfere the quorum sensing. This strategy involved any organism that is able in degrading quorum sensing signal molecules without inhibiting the pathogens growth. Since quorum sensing degraders do not disrupt the bacterial growth, it could reduce the risk of resistance due to the low selective pressure (Rutherford and Bassler, 2012).Furthermore, it allows the host's immune system to naturally recognize and destroy the pathogens.

In larviculture, live feed are necessary for first feeding because of their small mouth. The gut of the larvae is also not fully developed at the time of first feeding. Thus, the use of live feed may contribute to some exo-enzymes e.g., protease, lipase, amylase increment of productionsthat would aid in the feed digestion of the fish (Metges, 2000). Furthermore, larvae is attracted to moving prey than inert particles or artificial feed. One of the common live feed used in aquaculture is the brine shrimp, *Artemia*. In aquaculture, the nauplii of *Artemia* are generally the most commonly used live feed organisms. *Artemia* cyst is easy to obtain and providing live *Artemia* nauplii to grouper larvae (after the rotifer-feeding stage) still remains essential in commercial hatchery. Annually, thousands of metric tons of dry *Artemia* cysts are marketed globally for use in marine aquaculture. However, *Artemia* shows low biochemical composition, therefore, enrichment is necessary for first feeding of larvae (Sorgeloos *et al.*, 2001).

Interestingly, the live nauplii of brine shrimp (*Artemia* spp.) have also been used as mediums for carrying compounds of diverse nutritional and therapeutic value to larval stages of fish through a process known as bioencapsulation. *Artemia* bioencapsulated with lactic acid bacteria have been successfully introduced into orange-spotted grouper larvae with significant improvements in survival (Sun *et al.*, 2013).

A survey made by Tanjong Demong Marine Fish Production and Research Centre (PPIL), Terengganu revealed that *Vibrio alginolyticus*and *Vibrio harveyi* are the most virulent*Vibrio* isolated from infected grouper during disease outbreak (Ransangan *et al.*, 2012; Ali *et al.*, 2008). Although antibiotics have been used as one of the prevention method,the use of antibiotics have had restricted success since studies have shown that the pathogen has developed resistance against antibiotics (Hamza *et al.*, 2015). This condition showed that there is a need to discover another option against Vibrios. Given that there are researches that showed pathogenicity of *V. harveyi* is regulated by quorum

sensing in other organisms as being demonstrated by Yang and Defoirdt (2014), Natrah *et al.*(2011a) and Ruwandeepika *et al.*(2011) thus, in this study we focused on the impacts of quorum sensing on the virulence of *V. harveyi* towards tiger grouper larvae.

To our knowledge there is no study of bacterial QS on grouper larvae. Furthermore, the potential of live feed *Artemia* as the carrier of bacterial quorum sensing degrader for tiger grouper larvae was also investigated. The specific objectives of the study are:

1. To investigate the virulence of *Vibrio harveyi* and the impact of *V. harveyi* QS towards tiger grouper *E. fuscoguttatus* larvae.
2. To isolate and screen QS degraders from live feed *Artemia*.
3. To evaluate the effectiveness of the QS degraders encapsulated to *Artemia* towards tiger grouper *E. fuscoguttatus* larvae.

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