



UNIVERSITI PUTRA MALAYSIA

***DETECTION, PREVALENCE AND ANTIBIOTIC SUSCEPTIBILITY
PROFILE OF *Vibrio* SPECIES ISOLATED FROM *Epinephelus*
SPECIES IN PENINSULAR MALAYSIA***

NOR AMALINA BINTI ZULKIPLY

IB 2021 15



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By

NOR AMALINA BINTI ZULKIPLY

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of
Philosophy**

March 2021

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

DETECTION, PREVALENCE AND ANTIBIOTIC SUSCEPTIBILITY PROFILE OF *Vibrio* SPECIES ISOLATED FROM *Epinephelus* SPECIES IN PENINSULAR MALAYSIA

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March 2021

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Infection of *Vibrio* species (spp.) among groupers (*Epinephelus* spp.) is one of the most reported diseases since it affects groupers' production. Although it caused high mortality among groupers, a complete and up-to-date database on the prevalence and diversity of bacteria especially *Vibrio* spp. in groupers is not available in Malaysia. Thus, the objectives of this study were i) to characterize the bacterial diversity among cultured grouper and its culturing environment of seawater and sediment using a metagenomic approach, ii) to identify the prevalence and diversity of *Vibrio* spp. among cultured groupers in Peninsular Malaysia farms using the *pyrH* and *gyrB* genes, and iii) to determine the antimicrobial resistant profiles of *Vibrio* spp. In the metagenomic study, groupers' liver, sediment and seawater samples were analyzed based on the 16S rRNA V3-V4 regions using the Illumina MiSeq platform. A total of 270 cultured groupers collected from nine farms in Peninsular Malaysia were used in the isolation and identification of *Vibrio* spp. based on the *pyrH* and *gyrB* genes. Then, the antimicrobial resistant profiles of *Vibrio* spp. isolates were determined using seven antibiotics such as ampicillin, penicillin, bacitracin, erythromycin, tetracycline, streptomycin and vancomycin. Results of the metagenomics test were generated 801,383 sequence reads and 9,308 operational taxonomic units (OTUs). The sediment showed the most diverse bacterial communities since it revealed the highest OTUs (7,378) compared to seawater (1,763) and liver of grouper (167). From the OTUs, phylum Proteobacteria was observed in the sediment (60%), seawater (54%) and liver (32%). Phylum Firmicutes was found only in the sediment and liver. Phylum Actinobacteria, Bacteroidetes, Cyanobacteria, Planctomycetes, and Chloroflexi were observed in sediment and seawater. Genus *Vibrio* and *Photobacterium* of phylum Proteobacteria were the only genera shared by the sediment, seawater and liver. *Vibrio* (77% - 96%) was dominantly present in the sediment and seawater compared to *Photobacterium* (2% - 7%). Similar *Vibrio* OTUs (denovo951 and denovo43955) were found in

the sediment, seawater and liver indicating that there was a possible transmission of *Vibrio* between groupers and its surrounding environment. A study on the 270 cultured groupers collected from nine farms revealed that 380 *Vibrio* spp. were isolated from the pseudo-replicates of liver, spleen, and kidney of 195 (72%) cultured groupers. Results revealed that lesion was an early symptom of *Vibrio* infection since 82% of the cultured groupers were developed lesions on their skin, mouth and fins. A high number (25%) of the asymptomatic groupers were identified, thus, can be a *Vibrio* reservoir and threat to grouper farming. *Vibrio* spp. isolated from the groupers at the net cages was higher compared to the hatcheries. Analysis of the water quality is one of the contributor factors revealed that no correlation between the water quality parameters and increased number of *Vibrio* spp. at farms. Molecular analysis revealed that the *pyrH* gene was a superior phylomarker than the *gyrB* gene in identifying *Vibrio* spp. since it has high discriminatory power in differentiating species levels of *Vibrio*. Analysis of the phylogenetic tree of 380 *pyrH* sequences resulted in 13 *Vibrio* groups such as 28% of *V. owensii*, 25% of *V. parahaemolyticus*, 19% of *V. alginolyticus*, 14% of *V. vulnificus*, 3% of *V. rotiferianus* and *Vibrio* sp., 2% of *V. campbellii*, *V. mytili* and *V. furnissii*, and 1% of *V. harveyi*, *V. diabolicus*, *V. fluvialis* and *V. tubiashii*. Moreover, results revealed that groupers able to carry more than one *Vibrio* spp. simultaneously, thus, can enhance the mortality rate. *Vibrio* spp. also prone to infect the juvenile groupers rather than pre-adult groupers due to the immature immune systems developed. Analysis on the antimicrobial resistance profile suggested that ampicillin and penicillin G were ineffective in treating *Vibrio* infections since a high number (80%) of *Vibrio* spp. were resistant to both antibiotics. Effective monitoring on the administration of the bacitracin, erythromycin and vancomycin was required since 30% to 54% of the *Vibrio* isolates were found resistant to these antibiotics. Meanwhile, the resistance of *Vibrio* spp. to tetracycline and streptomycin was still low (14%). Most of the species included *V. parahaemolyticus*, *V. alginolyticus*, *V. rotiferianus*, *V. campbellii* and *V. diabolicus* were highly susceptible to both antibiotics. Multiple antibiotic resistance (MAR) index revealed that 88% of the *Vibrio* spp. had MAR index value of more than 0.2. Thus, the result indicated that the high number of *Vibrio* spp. were resistant to multiple antibiotics and continuously exposed to the antibiotics at the farms. Plasmid profiling results showed that 61% of *Vibrio* spp. were chromosomal-mediated with MAR index 0.37 and 39% were plasmid-mediated with MAR index 0.56. After the curing process, most of the *Vibrio*-positive plasmid were loss their resistance to antibiotics and the AMR index was reduced to 0.21. Thus, results indicated that the presence of problem can enhance the resistance of *Vibrio* spp. to antibiotics. In a conclusion, this study provided constructive documentation of bacterial communities in the groupers, sediment and seawater, prevalence of *Vibrio* infection among cultured groupers in Peninsular Malaysia, and occurrence of antibiotic resistance of *Vibrio* spp. presence in the cultured groupers.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENGESANAN, KELAZIMAN DAN PROFIL RINTANGAN ANTIBIOTIK
TERHADAP SPESIS *Vibrio* DIISOLAT DARI SPESIS *Epinephelus* DI
SEMENANJUNG MALAYSIA**

Oleh

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Jangkitan spesis *Vibrio* (spp.) di kalangan ikan kerapu (*Epinephelus* spp.) merupakan salah satu penyakit yang banyak dilaporkan kerana ia telah menjejaskan hasil pengeluaran ikan kerapu. Walaupun ia menyebabkan kadar kematian yang tinggi di kalangan ikan kerapu, namun, pengkalan data yang lengkap mengenai kelaziman dan kepelbagaian *Vibrio* di kalangan ikan kerapu di Malaysia masih belum didapati. Oleh itu, objektif kajian ini adalah, i) untuk mengenalpasti kepelbagaian bakteria yang terdapat di kalangan ikan kerapu yang ditenak, dan air laut serta enapan di sekitar kawasan ternakan menggunakan kaedah metagenomik, ii) untuk mengenalpasti kelaziman dan kepelbagaian *Vibrio* spp. di kalangan ikan kerapu yang ditenak di ladang ternakan Semenanjung Malaysia menggunakan gen *pyrH* dan *gyrB*, and iii) untuk menentukan profil kerentanan antimikrob di kalangan *Vibrio* spp. Dalam kajian metagenomik, sampel hati, enapan dan air laut dianalisis berdasarkan rantau 16S rRNA V3-V4 menggunakan platform Illumina MiSeq. Sebanyak 270 ikan kerapu yang ditenak di sembilan ladang ternakan sekitar Semenanjung Malaysia telah ditangkap dan digunakan untuk isolasi dan pengenalpastian *Vibrio* spp. berdasarkan gen *pyrH* dan *gyrB*. Kemudian, profil kerentanan antimikrob telah dilakukan terhadap isolat *Vibrio* spp. menggunakan tujuh antibiotik seperti ampisilin, penisilin, basitrasin, eritromisin, tetrasiklin, streptomisin dan vankomisin. Hasil keputusan daripada kajian metagenomik telah menghasilkan 801,383 urutan pembacaan dan 9,308 unit taksonomi operasi (OTUs). Enapan menunjukkan kepelbagaian komuniti bakteria terbanyak berbanding air laut dan hati ikan kerapu kerana ia telah mencatatkan bacaan OTUs tertinggi (7,378) berbanding dengan air laut (1,763) dan hati ikan kerapu (167). Berdasarkan OTUs, filum *Proteobacteria* telah dijumpai di dalam enapan (60%), air laut (54%) dan hati (32%). Filum *Firmicutes* hanya dijumpai di dalam enapan dan hati. Filum *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*,

Plancomycetes dan *Chloroflexi* telah dijumpai di dalam enapan dan air laut. Genus *Vibrio* dan *Photobacterium* daripada filum *Proteobacteria* merupakan satu-satunya genus yang hadir di dalam enapan, air laut dan hati. *Vibrio* (77% - 96%) lebih banyak dijumpai di dalam enapan dan air laut berbanding dengan *Photobacterium* (2% - 7%). *Vibrio* OTUs yang sama (denovo951 dan denovo43955) telah dijumpai di dalam enapan, air laut dan hati menunjukkan bahawa terdapat kemungkinan penularan *Vibrio* antara ikan kerapu dan persekitarannya. Kajian terhadap 270 ikan kerapu yang telah ditangkap dari sembilan ladang ternakan mendapati sebanyak 380 *Vibrio* spp. telah diisolat daripada 195 (72%) ekor ikan kerapu menggunakan hati, limpa dan buah pinggang secara ulangan-pseudo. Hasil kajian telah menunjukkan luka merupakan tanda-tanda awal terhadap jangkitan *Vibrio* kerana 82% daripada ikan kerapu mempunyai luka di bahagian kulit, mulut dan sirip. Kadar bilangan ikan kerapu tanpa simptom (25%) yang tinggi telah dikenalpasti sekaligus boleh menjadi tempat pengumpulan *Vibrio* dan ancaman kepada penternakan ikan kerapu. *Vibrio* spp. lebih banyak diisolat daripada ikan kerapu yang ditenak di dalam sangkar berbanding di dalam tangki. Analisis terhadap salah satu faktor penyumbang iaitu kualiti air telah menunjukkan bahawa tiada korelasi antara parameter kualiti air dan peningkatan jumlah *Vibrio* spp. di ladang. Analisa molekul telah menunjukkan bahawa gen *pyrH* merupakan filomarker yang lebih unggul berbanding gen *gyrB* dalam pengenalpastian *Vibrio* spp. kerana gen *pyrH* mempunyai kuasa diskriminasi yang tinggi dalam membezakan aras spesies *Vibrio*. Berdasarkan analisa filogenetik terhadap 380 jujukan *pyrH*, 13 kumpulan *Vibrio* spp. telah dikenalpasti iaitu 28% adalah *V. owensii*, 25% *V. parahaemolyticus*, 19% *V. alginolyticus*, 14% *V. vulnificus*, 3% *V. rotiferianus* dan *Vibrio* sp., 2% *V. campbellii*, *V. mytili* dan *V. furnissii*, dan 1% *V. harveyi*, *V. diabolicus*, *V. fluvialis* dan *V. tubiashii*. Selain itu, hasil kajian menunjukkan ikan kerapu yang ditenak berupaya membawa lebih daripada satu *Vibrio* spp. secara serentak, justeru, ia boleh meningkatkan kadar kematian. *Vibrio* spp juga lebih banyak menjangkiti ikan kerapu remaja berbanding ikan kerapu pra-dewasa kerana mempunyai sistem imunisasi yang masih belum matang. Analisa terhadap profil kerentanan antimikrob telah menunjukkan bahawa ampisilin dan penisilin G adalah tidak efektif untuk merawat jangkitan *Vibrio* kerana bilangan *Vibrio* spp. yang tahan kerentanan terhadap kedua-dua antibiotik adalah amat tinggi (80%). Pemantauan secara berkesan terhadap penggunaan basitrasin, eritromisin dan vankomisin amat diperlukan kerana telah terdapat 30% hingga 54% *Vibrio* isolat yang tahan kerentanan terhadap antibiotik tersebut. Manakala, kerentanan *Vibrio* spp. terhadap tetrasiklin dan streptomisin adalah masih rendah (14%). Kebanyakan spesies termasuk *V. parahaemolyticus*, *V. alginolyticus*, *V. rotiferianus*, *V. campbellii* dan *V. diabolicus* sangat terdedah kepada kedua-dua antibiotik. Indeks kerentanan antibiotik pelbagai (MAR) telah menunjukkan sebanyak 88% *Vibrio* spp. mempunyai nilai indeks MAR lebih daripada 0.2. Oleh itu, hasil keputusan mendapati bahawa bilangan *Vibrio* spp. yang tahan kerentanan terhadap pelbagai antibiotik adalah tinggi dan sentiasa terdedah kepada antibiotik di ladang ternakan. Keputusan profil plasmid telah menunjukkan bahawa 61% daripada *Vibrio* spp. merupakan mediasi-kromosom dengan indek MAR 0.37 dan 39% merupakan mediasi-plasmid dengan indek MAR 0.56. Selepas proses "curing", kebanyakan *Vibrio*-positif plasmid telah kehilangan kerentanan terhadap antibiotik dan indek MAR telah menurun kepada 0.21. Lantas, keputusan ujian menunjukkan bahawa kehadiran plasmid yang membawa kerentanan-antibiotik berupaya meningkatkan daya tahan *Vibrio*

spp. terhadap antibiotik. Sebagai kesimpulan, kajian ini menyediakan dokumentasi yang konstruktif terhadap komuniti bakteria di dalam kerapu, enapan dan air laut, kepelbagaian jangkitan *Vibrio* dikalangan ikan kerapu yang ditanam di Semenanjung Malaysia, dan kehadiran *Vibrio* spp. yang tahan kerentanan terhadap antibiotik di kalangan ikan kerapu yang ditanam.



ACKNOWLEDGEMENTS

Alhamdulillah. I would like to express appreciation and gratitude to my supervisor, Assoc. Prof. Dr. Ina Salwany Md Yasin for her guidance, patience and support in my studies. Huge thanks also go to my co-supervisor, Prof. Dr. Mohd Zamri Saad, Assoc. Prof. Dr. Mohammad Noor Amal Azmai and Dr. Dzarifah Zulperi who are very compassionate, encouraging, and supportive.

I wish to express thanks to all my friends especially Dr Santha Silvaraj, Diyana Nadhirah, Fatin Amirah, Amatul Samahah Md. Ali, Dr Nurliyana Mohamad, Jumriah, Dr Aslizah Mohd Aris, Dr Nur Hidayu Al-Saari, Dr Ida Muryany Md Yasin, Yong Kit Chin, Lee Jing Yie, other students and academic staffs of the Biotechnology Aquaculture Laboratory, Faculty of Aquaculture, UPM and Laboratory of Marine Biotechnology (MARSLAB), Institute of Bioscience, UPM for the great support and endless motivation. I really appreciate all your help.

I would like to thank my husband, Muhd Fairul Azman Aziz, my mother, Latifah Awang and my lovely daughter, Nur Farina Aisyah. No words can be written to picture their endless love, motivation and emotional supports. Thanks to all my family members, my mother-in-law, Sariah Taib, my father-in-law, Aziz Mahmud and to my siblings, Nur Adilah, Mohammad Amirul Faiz, Mohammad Khairul Amilin, Muhammad Amru Irfan, Nurul Etipah, Mohamad Faizatul Ikmal and Ali Jalaluddin. Thank you for being supportive and encourage me to be a better person.

This research was financially funded by the Ministry of Education Malaysia via Higher Institution Centre of Excellent (HiCoE) grant. Thank you also goes to Ministry of Education Malaysia for providing me the MyBrain 15 (MyPhD) fellowship to support my study. I would also thank the Universiti Putra Malaysia for the Geran Putra-Inisiatif Putra Siswazah (GP-IPS) that funding part of this research.

Thank you very much.

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

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LIST OF ABBREVIATIONS

α	Alpha
β	beta
AMR	antimicrobial resistance
BLASTn	nucleotide Basic Local Alignment Search Tool
bp	base pair
CI	confidence interval
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
ddNTPs	dideoxyribonucleotide triphosphates
DO	dissolved oxygen
DOF	Department of Fisheries Malaysia
FAO	Food and Agriculture Organization of United Nations
GTR	general time reversible
LDC	lysine decarboxylase
M	molar
mA	milliampere
MAFFT	Multiple Alignment of Fast Fourier Transform
MAR	multiple antibiotic resistance
MgCl ₂	magnesium chloride
MHA	Muller-Hinton agar
ML	maximum likelihood
mM	millimolar
MT	million tonnes
NaCl	sodium chloride

NGS	next gene sequencing
NJ	neighbour-joining method
nM	nanomolar
OPNG	ortho-nitrophenyl- β -galactoside
OTUs	operational taxonomic units
PC	principal coordinate
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
pM	picomolar
ppt	part per thousand
QC	quality control
QIIME	Quantitative Insights into Microbial Ecology
RNA	ribonucleic acid
RT	room temperature
TAE	tris-acetic EDTA
TCBS	thiosulfate citrate bile salts-sucrose
TGGG	Tiger grouper Giant grouper (Hybrid grouper)
TSI	triple sugar iron
U	unit
UV	ultra violet
V	voltan

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Recently, total global fish production has increased to meet the food demand for human consumption. According to the Food and Agriculture Organization of United Nations (FAO), the total global fish production had increased from 148 million tones (MT) in 2010 to 179 MT in 2018 (FAO, 2014; FAO, 2020). In 2018, 54% of total production was contributed by the capture fishery, while the remaining 46% came from the aquaculture sector. Even though the total production from capture fisheries is still dominant, it has shown a stagnant production value since the late 1980s. Meanwhile, aquaculture rose to 527% in global aquaculture production from 1990 to 2018 and is responsible for the sustainability of fish supply in the near future (FAO, 2018, FAO, 2020).

Department of Fisheries Malaysia (DOF) reported that the production of marine fish recorded in 2019 was 1.87 MT, with a value of RM 15.26 billion (DOF, 2020). From that, 1.5 MT contributed by the capture fishery, 411 782 tones by aquaculture, and 5 569 tones by inland fishery sectors (DOF, 2020). The production was increased by 1.21% in 2019 compared to 2018 (1.85 MT) and indicates that the Malaysian's appetite for fish products shows no sign of slowing (DOF, 2020). Yusoff (2015) reported that the index of fish consumption is expecting to increase from 53.1 kg in 2011 to 61.1 kg in 2020. Unfortunately, declining in the total production of the aquaculture sector has been reported from 2014 (520 515 tones) to 2019 (411 782 tones) (DOF, 2020).

The main setback in aquaculture is the disease outbreaks among finfishes, mollusks, crustaceans, and other aquatic animals. Diseases can cause a significant problem to the development of this sector and lead to high mortalities, loss of food supply, and severe economic losses worldwide (Austin, 2010). As example, 15-20% of mortality rate was recorded among cultured fish that lead to USD 61.6 million loss (Norwegian Directorate of Fisheries, 2020). In Malaysia aquaculture, diseases of vibriosis, streptococcosis, motile aeromonas septicemia, piscine tuberculosis and edwardsiellosis were reported among the cultured fish (Altinok et al., 2006, Hardi et al., 2018, Wang et al., 2020a).

The development of diseases among the cultured fish were caused by the environmental and mechanical factors such as poor water quality, stress, overcrowding fish, improper fish nutrition, pollution and predators (Ina-Salwany et al., 2019). However, presence of microorganisms such as bacteria, virus, archaea in the fish environments can be beneficial in improving water quality,

provide fish nutrient, and prevent pollution (Onianwah et al., 2018). For examples, *Pseudomonas* and *Bacillus* spp. that presence in the seawater play roles in controlling heavy metal, oxygen and nitrogen (Cardoso et al., 2012). Phytoplankton, microalgae and bacteria can help in food digestion and provide nutrient like vitamin and enzyme to the fish (Shukla et al., 2020). Thus, balance of the non-pathogenic and pathogenic microorganisms' community inside the host, seawater and sediment can help in controlling water quality, pollution and providing nutrient as well as able to reduce fish diseases.

Epinephelus spp. (groupers) has been dominating the marine aquaculture production in Malaysia due to the high export value (RM 282 172) (DOF, 2020). However, vibriosis caused by *Vibrio* spp. was a main disease among the groupers (Chuah, 2001). First outbreak of vibriosis in Malaysia aquaculture was occurred in 1990 involving *Epinephelus* spp., *Lates calcafirer* (sea bass), and *Lutjanus* spp. (red snapper) that caused USD 7.4 million loss (Shariff, 1995). In addition, *Vibrio* infection among cultured fish leads to global loss of USD 1.6 billion in 2011 (Akazawa et al., 2014). *Vibrio vulnificus*, *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, and *V. anguillarum* are the most frequently isolated marine *Vibrio* spp. and have been associated with a large-scale loss of aquatic animals (Pujalte et al., 2003; Chatterjee and Haldar, 2012; Haenen et al., 2014, Alipiah et al., 2016).

Antibiotics were frequently used as a treatment of diseases among the cultured fish such as ampicillin, tetracycline, vancomycin and others (Deng et al., 2020). However, presence of the multiple antibiotic resistance (MAR) among bacteria was reduced the efficiency of the antibiotics in disease treatment and becoming the main threat to the aquaculture sector (Lesley et al., 2011; Al-Othubi et al., 2014). Thus, proper administration of the antibiotics was required in prevent increasing number of MAR among bacteria.

1.2 Problem statements

Grouper fish remains one of the important economic driven fish in Malaysia due to the high demand for local and export markets (7 370 tones) with a high market price (RM 282 172) compared to other cultured fish (DOF, 2020). However, disease among groupers were frequently reported including *Vibrio* infection (Mahmud et al., 2010, Liu et al., 2016, Nishiki et al., 2018, Sumithra et al., 2019). In addition, in 2016, an outbreak among juvenile hybrid groupers (*Epinephelus polyphkadion* × *E. fuscoguttatus*) caused by *Vibrio harveyi* and *V. alginolyticus* was reported in Selangor, Malaysia (Mohamad et al., 2019b). Even though many studies reported on the disease infection among cultured groupers in Malaysia aquaculture, information related with bacterial distribution and diversity among cultured groupers in Malaysia aquaculture was still limited.

As known, interaction between microorganisms, host and environments were important in the functioning of ecosystem (Engering et al., 2013). The bacteria presence in the fish and its surrounding environment can reflect the condition and safety of the aquatic environments (Novoslavskij et al., 2016). Moreover, the pathogen in the environments can be transmitted to the fish during unfavorable stress conditions (Mohamad et al., 2019a). Thus, metagenomics based on the analysis of microorganism genomic DNA directly from host and environments can fill a knowledge gap in providing informative knowledge on the microbial diversity and function within the ecosystem (Kaviarasu and Sudhan, 2018).

There also lack of information available regarding the prevalence and distribution of *Vibrio* spp. among cultured groupers surrounding Malaysia. Limited studies have been conducted, but covered one or two *Vibrio* spp. and in limited geographical areas such as in Selangor only (Noorlis et al., 2011, Letchumanan et al., 2015a, Mohamad et al., 2019b). Thus, information regarding the prevalence and distribution of *Vibrio* spp. in farms surrounding Malaysia was necessary to provide better understanding the epidemiology of *Vibrio* infection over a geographical region.

Moreover, there was no proper database on the early diagnosis in identification of *Vibrio* spp. among cultured groupers. Combination of gross observation of disease symptom, water quality measurement and diagnostics tools are important to provide a rapid and effective diagnosis for early identification and treatment of disease (Burge et al., 2016). Thus, information regarding the early symptoms, age and size of cultured groupers, fluctuation of water quality, combined with a high discriminatory diagnostic tool could be useful in the aquaculture industry.

Lack of up-to-date antibiotic resistance of *Vibrio* spp. among cultured groupers is also the major health challenges. The information on the antibiotic resistance and resistant genetic determinants is important because knowledge gaps in how serious occurrence of resistance strains among *Vibrio* spp. in the groupers farms. Since antibiotics are commonly used to prevent and treat of disease in fish, excessive usage of antibiotics leads to increase of resistance strains (Letchumanan et al., 2015a, Agoba et al., 2017). Thus, lack of information on the resistance strains leads to misuse of antibiotics among the farmers, ineffective treatment of diseases and potential of huge loss of cultured fish.

1.3 Significance of the study

Up to this point, the occurrence of *Vibrio* spp. among groupers has not been documented well in Malaysia. Therefore, this study will provide up-to-date and informative database on the diversity of pathogenic and non-pathogenic bacteria presence in the groupers, seawater and sediment, prevalence and taxonomic

distribution of *Vibrio* spp. among the cultured groupers in Peninsular Malaysia farms, and current occurrence of the antimicrobial resistant pattern among *Vibrio* spp. Understanding the diversity of microorganisms in the groupers and surrounding environments via metagenomics will be able to provide the first step towards exploring proper management of cultured groupers in the farms.

Knowing the disease prevalence and distribution can help farmers, researchers and policy makers to develop control measure of diseases in fish and farms. Moreover, information on the contribution of antibiotic usage in farms, potential risks on environment and fish health, the availability of antibiotic resistance strains and presence of genetic determinants are useful for regulations to control practices on the farms and reduce the magnitude of the antibiotic resistance among *Vibrio* spp. Thus, the finding from this study can contribute to the fish health management plan.

1.4 Objectives of the study

This study was carried out with the following objectives:

1. To characterize the bacterial diversity among groupers (*Epinephelus* spp.) and its culturing environments, particularly the sediment and seawater with a metagenomics approach.
2. To identify the prevalence and diversity of *Vibrio* spp. from cultured groupers (*Epinephelus* spp.).
3. To determine the taxonomic distribution of *Vibrio* spp. among cultured groupers (*Epinephelus* spp.) using phylogenetic analysis of *gyrB* and *pyrH* genes.
4. To determine the antibiotic resistant patterns, plasmid profiling, and plasmid curing of *Vibrio* spp. from cultured groupers (*Epinephelus* spp.).

1.5 Hypotheses of the study

The hypotheses of this study are as below:

Hypothesis 1:

H₀: The cultured groupers did not have similar bacterial diversity as the sediment and seawater.

H₁: The cultured groupers have similar bacterial diversity as the sediment and seawater.

Hypothesis 2:

H₀: The *Vibrio* spp. was not present in the cultured groupers sampling at farms in Peninsular Malaysia.

H₁: The *Vibrio* spp. was present in the cultured groupers sampling at farms in Peninsular Malaysia.

Hypothesis 3:

H₀: The *gyrB* or *pyrH* genes were not able to identify the taxonomic distribution of *Vibrio* spp. among cultured groupers using phylogenetic analysis.

H₁: The *gyrB* or *pyrH* genes were able to identify the taxonomic distribution of *Vibrio* spp. among cultured groupers using phylogenetic analysis.

Hypothesis 4:

H₀: The *Vibrio* spp. isolated from the cultured groupers were not resistant to the antibiotics tested.

H₁: The *Vibrio* spp. isolated from the cultured groupers were resistant to the antibiotics tested.

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