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DEVELOPMENT OF DUPLEX POLYMERASE CHAIN REACTION FOR DETECTION OF *Vibrio alginolyticus* INFECTION IN MARINE FISH

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By

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DEVELOPMENT OF DUPLEX POLYMERASE CHAIN REACTION FOR DETECTION OF *Vibrio alginolyticus* INFECTION IN MARINE FISH

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Vibrio alginolyticus species are among key pathogens that causes Vibriosis in a marine culture that can eventually lead to problems in food safety and bacterial disease management.In this study, an effective duplex PCR was developed for simultaneous and rapid detection species specific to*Vibrio alginolyticus* by using *gyr*B and collagenase genes. The *gyr*B and collagenase genes were amplified using purified genomic DNA of *V. alginolyticus* and other *Vibrio* species with an estimated size of 337 bp and 737 bp respectively. The *gyr*B gene was specifically detected for all *Vibrio* sp and collagenase gene was specifically detected only for *V. alginolyticus.* The PCR products were cloned and sequenced. The *Vibrio* species and non-*Vibrio* species were conducted to specificity test, and it revealed that the primer sets were specific to *V. alginolyticus* and *Vibrio* species only. The sensitivity test was accomplished with various DNA concentrations of *V. alginolyticus* (100pg to 1pg), resulting in the lowest value of 1pg. The developed duplex PCR was also tested against artificial infection of groupers that were injected with 10^{10}CFU/ml of *V. alginolyticus*, *V. parahaemolyticus*, and *A.hydrophila.* At 24 hours post-injection, the organs (liver, spleen, and kidney) were homogenized and a colony bacterium on TCBS agar was used in duplex PCR assay.All pairs of primers were shown to be specific and sensitive to *V. alginolyticus* and able to amplify collagenase and *gyr*B genes at 737 bp and 337 bp respectively. The result of artificially infected groupers showed that the duplex PCR was able to detect *V. alginolyticus*. The efficacy of this duplex PCR assay was confirmed from collected diseased groupers from the aquatic environment. It showed that duplex PCR assay was able to amplify *gyr*B

gene for only 12 samples of *Vibrio* species, however no amplification was observed for collagenase gene. Therefore, these 12 samples were sent for sequenced and ran for BLASTn analysis. Based on the analysis, only two isolates were correctly identified as *V. alginolyticus* strains (BK5G3 and BK3G1). The extracted DNA of *V. alginolyticus* strains (BK5G3 and BK3G1) were subjected to duplex PCR assay and the result showed no amplification of collagenase gene. To confirm the presence of collagenase gene, the experiment was further carried out using *Lates calcarifer* as a fish model. However, no amplification was observed for collagenase gene. Therefore, the newly developed duplex PCR assay is able to be used *in-vivo* but unable to be apply in *in-vitro* study. Newly developed duplex PCR assay is, therefore, possible to use in food security analysis and disease detection of aquatic animals.

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

PEMBANGUNAN TINDAK BALAS BERANTAI POLIMERASE DUPLEK UNTUKPENGESANAN JANGKITAN *Vibrioalginolyticus* DALAM IKAN MARIN

Oleh

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Spesis *Vibrio alginolyticus* adalah antara patogen utama yang menyebabkan Vibriosis dalam kultur marin yang akhirnya boleh membawa masalah kepada keselamatan makanan dan pengurusan penyakit bakteria. Dalam kajian ini, PCR duplek yang berkesan telah dibangunkan untuk pengesanan serentak dan spesifik terhadap*Vibrio alginolyticus* dengan menggunakan *gyr*B dan kolagenase gen. *Gyr*B dan kolagenase gen diamplifikasi menggunakan DNA genomik yang ditulenkandari *V. alginolyticus* dan spesies *Vibrio* yang lain dengan anggaran saiz masing-masing 337 bp dan 737 bp. Gen *gyr*B secara khusus dikesan untuk semua *Vibrio* sp dan kolagenase secara khusus dikesan hanya untuk *V. alginolyticus*. Produk PCR telah diklon dan dijujukan. Spesies *Vibrio* dan spesies bukan *Vibrio* telah digunakan untuk ujian spesifik, dan ianya mendedahkan bahawa set primer adalah khusus untuk spesies *V. alginolyticus* dan *Vibrio* sahaja. Ujian kepekaan dicapai dengan pelbagai kepekatan DNA *V. alginolyticus* (100pg hingga 1pg), menghasilkan nilai terendah 1pg. PCR duplek yang dibangunkan juga diuji terhadap jangkitan artifisial ikan kerapu yang disuntik dengan 10^{10} CFU / ml *V. alginolyticus, V. parahaemolyticus,* dan *A. hydrophila*. Pada 24 jam pasca-suntikan, organ-organ (hati, limpa, dan ginjal) telah dihomogenat dan bakteria koloni di atas TCBS agar digunakan dalam ujian PCR dupleks. Semua pasangan primers ditunjukkan khusus dan sensitif terhadap *V. alginolyticus* dan mampu mengamplifikasikan gen kolagenase dan *gyr*B pada 737 bp dan 337 bp masing-masing. Keputusan dari ikan yang dijangkiti secara artifisial menunjukkan bahawa PCR duplek dapat mengesan *V.*

alginolyticus. Keberkesanan ujian PCR duplek ini telah disahkan melalui pengumpulan kerapu berpenyakit dari persekitaran akuatik. Ianya menunjukkan bahawa PCR duplek assai dapat mengamplifikasikan *gyr*B gen hanya untuk 12 sampel spesies *Vibrio,* namun tiada amplifikasi diperhatikan untuk gen kolagenase. Oleh itu, 12 sampel telah dihantar untuk penjujukan dan analisis BLASTn dijalankan. Berdasarkan analisis, hanya dua isolat yang berjaya dikenal pasti sebagai strain *V. alginolyticus* (BK5G3 dan BK3G1). DNA yang diekstrak dari strain *V. alginolyticus* (BK5G3 dan BK3G1) digunakandalamPCR duplekassaidan menunjukkan tiada amplifikasi dari gen kolagenase. Untuk mengesahkan kehadiran gen kolagenase, eksperimen ini terus dijalankan dengan menggunakan *Lates calcarifer* sebagai model ikan. Walau bagaimanapun, tiada amplifikasi dihasilkan bagi gen kolagenase. Oleh itu, ujian PCR dupleks yang baru dibangunkan ini mampu digunakan di dalam *in-vivo* tetapi tidak dapat digunakan dalam kajian *in-vitro*. Oleh itu, pembangunan PCR duplek assai yang baru ini mmpu di gunakan sebagai analisis keselamatan makanan dan pengesanan penyakit pada haiwan akuatik.

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

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AI	Auto inducer
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CaCl	Calcium chloride
CFU	Colony forming units
dATP	Deoxyadenosine triphosphate
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
hrs	Hours
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IUCN	International Union for Conservation of Nature
LB	Luria Bertani
Min	Minute
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MS-222	Tricaine methanesulfonate solution
NaCl	Sodium chloride
OD	Optical density
OMP	Outer membrane protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pmol	Picomole
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
s	Second

TBE	Tris-boric EDTA
TCBS	Thiosulfate-citrate-bile salts-sucrose
TSA	Tryptic soy agar
TSB	Tryptic soy broth
v/v	Volume per volume
w/v	Weight per volume
x g	Multiples of gravitational acceleration

CHAPTER 1

INTRODUCTION

Aquaculture sector today remains a growing and important protein based food productions to cater growing human population. The production of world fish culture had expanded at an average annual rate from 6.2% in the year 2000 until 2012 (9.5% in 1990- 2000) from 32.4 million up to 66.6 million tonnes. World aquaculture can be considered into two types; land aquaculture and marine culture. The growth in land aquaculture has overtaken mariculture growth contributing to 50% its fish production in 1980 and increasing to 63% in 2012 (FAO, 2014).

Throughout the global world aquaculture, the sector has provided one-third of the total food fish supply. At the national level, the production of marine fish from Malaysian waters was 1, 482, 899 tonnes immaculate the value of RM 8.336 billion in 2013 whereas the production had falloffs by 1.67 % to 1, 458, 128 tonnes immaculate with a value of RM 8785 billion in 2014 (Dof, 2016). Index of fish consumption had increase from 53.1 kg in year 2011 and is expected to be 61.1 kg in year 2020 which placed Malaysia one of the highest fish consumers in the world (Yusoff, 2015).

Similar to other farming sectors, the occurrence of infectious disease was caused by fungi, bacteria, parasites and other prominent pathogens increased as aquaculture activities intensified and expand. In numerous countries, diseases had become one of the main constrain in cultured aquatic organism, inhibiting both economic and social development (Bondad-Reantaso et al., 2005).There are two ways in which marine fish infectious diseases can impact a species' economic values.

Decreasing potential catch is due to increase in mortality, slower growth, immune defences infested by hosts, and host reactions to infection that can harm both hosts and infectious agents. Secondly, the fish's taste was off-flavour, had bad appearance and risks to human health (Lafferty et al., 2015).

Water quality is the main factor in aquaculture system for disease management. The non-optimum water physic-chemical parameters such as dissolved oxygen, pH, salinity, ammonia, temperature and poor management practice like overfeeding, inadequate nutrition and overcrowding causes stress to fish cultured thus make them susceptible to disease outbreaks (Boyd & Tucker, 1998; Zamri-Saad et al., 2014). Disease

management aspects should be taken seriously, since intensive fish culture areas gave harmful effects towards water quality on those areas (Gorlach-Lira et al., 2013).

The fast growing marine fish such as groupers (*E. fuscoguttatus*) has become farmers' choice for their intensive aquaculture. However, warming of marine and saline inland waters is likely to augment larger amounts of *Vibrio* populations which increased the risk of infection (Roux et al., 2015). Vibriosis has caused the farmers to struggle with losses due to mass mortalities of fry and fingerlings.The common clinical signs related to Vibriosis in groupers including dark skin, pale gills, hemorrhagic around mouth and skin ulceration (Sarjito et al., 2009)

In recent times, bacterial diseases have caused the major mortalities in various fish and shellfish species over all over geographical area (Saeed, 1995; Zhang & Austin, 2000).Vibriosis is a major cause that is related to bacterial disease in mariculture system (Bondad-Reantaso et al. 2005; Zhang et al. 2007; Ponprateep at al. 2009). *Vibrio* infections are acquired through ingestion or through an open wound (Iwamoto et al., 2010). The pathogens frequently associated with mass mortality during outbreaks include *V. alginolyticus, V. parahaemolyticus, V. harveyi,* and *V. anguillarum* (Alipiah et al., 2016). *Vibrio alginolyticus* is exists in marine surroundings, coastal and aquatic atmospheres (Narracci et al., 2013) with wide-reaching distribution. It is also vastly abundant and usually takes over *Vibrio* communities (Lai et al., 2004; Schets et al., 2010; Jones et al., 2013).It has been reported that *V. alginolyticus* is an associated agent with Vibriosis disease that caused mass mortality in the cultivation of yellow croakers*(Pseudosciaena crocea)*and economic losses(Yan et al., 2007). However the usage of PCR method in identifying *V. alginolyticus* is time consuming and can cause misidentification of the emergence of Vibrios species.

Molecular DNA based diagnostic assay, was based on polymerase chain reaction (PCR), have been deliberated and established for fast and accurate identification of *Vibrio* species.This protocol gives comparative benefitsto match usual microbiologicalculture procedures (Kaysner & DePaola, 1998; Jones et al., 2012). The polymerase chain reaction (PCR) was developed and established for speedy detection of *V. alginolyticus* (Robert-Pillot et al. 2002; Liu et al. 2004).Fast and efficient method for detection of this pathogenic *V. alginolyticus* is important in controlling and investigating Vibriosis outbreaks. The aim of this project is to develop duplex PCR assay which can be used in detection of *Vibrio* and *V. alginolyticus* species in single reaction and finally to give a precautionary measures against Vibriosis diseases associated with *V. alginolyticus*. In developing duplex polymerase chain

reaction (PCR) assay for diagnosis of *V. alginolyticus* in marine aquaculture. Three objectives of study were set. The three objectives were:

1. To clone and sequence *gyr*B and collagenase genes of *Vibrio alginolyticus* strain VA2 as gene marker for assay development.
2. To develop duplex PCR assay by using *gyr*B and collagenase genes for *V. alginolyticus* detection.
3. To assess the efficacy of developed duplex PCR assay for identification of *V. alginolyticus* in naturally and artificial infected fish.

Hypotheses of this study were:

H_o: The efficacy of new developed duplex PCR could be used and identify *V. alginolyticus* in naturally and artificial infected fish.

H_A: The efficacy of new developed duplex PCR could not be used and unable to identify *V. alginolyticus* strain in naturally and artificial infected fish.

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BIODATA OF STUDENT

Diyana Nadhirah was born in Shah Alam, Selangor in 20th May 1990 and grew up in Temerloh, Pahang. She began her primary education at Sekolah Kebangsaan Paya Taram from 1997 to 2002 and was selected to continue her secondary education at Sekolah Menengah Kebangsaan Kerdau, Temerloh for three years from 2003 to 2005. She was transferred into Sekolah Menengah Kebangsaan Temerloh Jaya for one year and after that she was transferred into Sekolah Menengah Kebangsaan Temerloh and had graduated on 2007. Then, she attended a two-year matriculation program at Johor Matriculation College (KMJ) before entering Universiti Putra Malaysia (UPM) in Serdang in 2010. There, she studied Agriculture, majored in aquaculture for four years. In 2014, she graduated from UPM with Bachelor's degree with honors.

In 2014, she commenced the Master of Science degree in the field of Aquaculture under the supervision of Dr. Ina Salwany Md Yasin at the Faculty of Agriculture, Universiti Putra Malaysia. She was involved in a research project to study the development of duplex polymerase chain reaction (PCR) for the diagnosis of *Vibrio alginolyticus* infection for marine aquaculture.

LIST OF PUBLICATIONS

Diyana-Nadhirah, K.P., and M.Y. Ina-Salwany. (2016). Molecular Characterization of 16S rRNA and Internal Transcribed Spacer (ITS) Regions of *Aeromonas* spp. Isolated from Cultured Freshwater Fishes in Malaysia.Int.J.Curr.Microbiol.App.Sci. 5(9): 431-440. doi: http://dx.doi.org/10.20546/ijcmas.2016.509.046

Rabi'atul, A. S, Diyana-Nadhirah, K. P., Nehlah, R., Saleema, M.,Zarirah.Z., & Ina-Salwany, M.Y. (2015). Internal Transcribed Spacer (ITS) Gene Sequencing and Pathogenicity Study of *Aeromonas veronii bv. sobria* Strain KTG3SB in Juvenile Red Tilapia. *National Seminar on Advances in Fish Health 2015*.4th – 5th February 2015, Kajang, Selangor, Malaysia.

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