ANTIBIOTIC RESISTANCE BACTERIA IN AQUACULTURE SOURCES IN JOHOR, MALAYSIA

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DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

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ABSTRACT

The fast-growing aquaculture sector has served as the reservoir in promoting the emergence of antibiotic resistance mechanisms. This study aimed to isolate and identify the bacteria from aquaculture sources and water samples followed by identification of the multiple antibiotic resistance (MAR) bacteria. The fishes and water samples from Fisheries Research Institute (FRI), Kukup, Pulai, Pendas Laut, Sungai Melayu and Kong Kong rivers were collected and subjected to both phenotypic and genotypic identification via 16S rRNA sequencing and phylogenetic analysis. Colony morphology and Gram-stain appearances of the bacterial isolates were observed as preliminary identification. Antibiotic susceptibility test via disc diffusion was performed for each of the bacterial isolates. In this study, a total number of 133 bacterial isolates were obtained and it was discovered that the antibiotic resistance character occurred in 63 bacterial isolates. From 63 isolates, 90.48% were resistant to rifampicin, 80.95% to ampicillin and 65.08% to sulphafurazole. High number (26.98%) of MAR bacterial isolates were isolated from FRI, suggesting that the origin of the isolates to be of high antibiotic usage. From 63 MAR isolates, 61 of them showed different characteristics in terms of antibiotic resistance and phenotypic test, indicates these isolates were from different strains. The 61 different MAR strains were subjected to 16S rRNA sequencing and the findings revealed that majority of the bacteria species obtained were *Bacillus* spp. (*Bacillus tropicus, Bacillus proteolyticus, Bacillus paramycoides, Bacillus cereus and Bacillus toyonensis*), *Pseudomonas* spp. (*Pseudomonas songnenensis, Pseudomonas pseudoalcaligenes, Pseudomonas plecoglossicida* and *Pseudomonas hibiscicola*), *Aeromonas* spp. *(Aeromonas caviae, Aeromonas hydrophila* and *Aeromonas rivipollensis*), *Acinetobacter* spp. (*Acinetobacter johnsonii* and *Acinetobacter modestus*) and *Enterobacter* spp. (*Enterobacter cloacae* and *Enterobacter xiangfangensis*). The phylogenetic analysis in each location suggested that the bacteria strains obtained in this study were closely related to the strains aligned that obtained from National Center for Biotechnology Information (NCBI) according to the neighbour-joining phylogenetic tree analysis, but with the low bootstrapping number, they cannot be assigned accurately to a certain species due to its low discrimination. This study revealed the imprudent use of antibiotics in aquaculture may pose high risk of antibiotic resistance and is crucial to raise public awareness on it. Therefore, there is a need to control the usage of antibiotics in aquaculture to avert the occurrence of multiple antibiotic resistant bacteria and contribute to the antibiotic prescription policies in this country.

ABSTRAK

Sektor akuakultur yang berkembang pesat telah berfungsi sebagai tempat meningkatnya kemunculan mekanisma kerintangan antibiotik. Kemunculan kerintangan antibiotik akan menyebabkan ancaman kepada populasi manusia serta jangkitan bakteria umum yang sukar dirawati. Kajian ini bertujuan untuk mengasing dan mengenal pasti bakteria daripada sumber akuakultur dan sampel air diikuti dengan pengesahan bakteria berbilang kerintangan antibiotik (MAR). Sampel ikan dan air dari Institut Penyelidikan Perikanan (FRI), Kukup, Pulai, Pendas Laut, Sungai Melayu dan Kong Kong telah dikumpulkan dan tertakluk kepada pengenalpastian fenotip dan genotipik melalui penjujukan 16S rRNA dan analisis filogenetik. Morfologi koloni bakteria dan penampilan Gram-stain dari isolat bakteria diperhatikan sebagai pengenalan awal. Ujian kerintangan antibiotik melalui kaedah resapan cakera dan dilakukan untuk setiap isolat bakteria. Dalam kajian ini, sejumlah 133 isolat bakteria telah diperoleh dan didapati bahawa sifat kerintangan antibiotik berlaku pada 63 isolat bakteria. Secara keseluruhannya untuk kerintangan antibiotik, 90.48% daripada isolat bakteria MAR tahan terhadap rifampicin, 80.95% terhadap ampicillin dan 65.08% kepada sulfatfurazol. Jumlah yang tinggi iaitu sebanyak 26.98% daripada bakteria MAR diperolehi dari FRI, menunjukkan bahawa tempat asal isolat telah menjadi tempat penggunaan antibiotik yang tinggi. Dalam 63 isolat MAR, 61 darinya menunjukkan ciri yang berlainan dari segi kerintangan antibiotik dan ujian fenotopik, yang bermaksud isolat ini adalah dari strain yang berlainan. 61 MAR bakteria tertakluk kepada perjujukan 16S rRNA dan penemuan menunjukkan bahawa majoriti spesies bakteria yang diperolehi ialah *Bacillus* spp. (*Bacillus tropicus, Bacillus proteolyticus, Bacillus paramycoides, Bacillus cereus* dan *Bacillus toyonensis*), *Pseudomonas* spp. (*Pseudomonas songnenensis, Pseudomonas pseudoalcaligenes, Pseudomonas plecoglossicida* dan *Pseudomonas hibiscicola*), *Aeromonas* spp. *(Aeromonas caviae, Aeromonas hydrophila* dan *Aeromonas rivipollensis*), *Acinetobacter* spp. (*Acinetobacter johnsonii* dan *Acinetobacter modestus*) dan *Enterobacter* spp. (*Enterobacter cloacae* dan *Enterobacter xiangfangensis*). Analisis filogenetik di setiap lokasi mencadangkan bahawa strain bakteria yang diperolehi dalam kajian ini adalah berkaitan erat dengan strain sejajar yang diperolehi daripada Pusat Kebangsaan Maklumat Bioteknologi (NCBI) mengikut analisis kejiranan terhubung pokok filogenetik, tetapi dengan jumlah bootstrapping yang rendah. Oleh itu, mereka tidak boleh ditentukan secara tepat kepada spesies tertentu disebabkan diskriminasi yang rendah. Kajian ini mendedahkan penggunaan antibiotik yang kurang bijak dalam akuakultur boleh menimbulkan risiko kerintangan antibiotik dan amat penting untuk meningkatkan kesedaran orang ramai terhadapnya. Oleh itu, keperluan untuk mengawal penggunaan antibiotik dalam akuakultur untuk mengelakkan berlakunya bakteria kerintangan antibiotik berbilang dan menyumbang kepada dasar preskripsi antibiotik di negara ini.

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LIST OF ABBREVIATIONS

AMR	-	Antimicrobial Resistance
µg	-	Micro-gram
µL	-	Microliter
µM	-	Micromiter
AAC	-	Acetyltransferase
ANT	-	Nucleotidyltransferase
APH	-	Phosphotransferase
ARB	-	Antibiotics Resistance Bacteria
ARG	-	Antibiotic Resistance Gene
BLASTn	-	Nucleotide-nucleotide Basic Local Alignment Search Tool
bp	-	basepair
BRIC	-	Brazil, Russia, India and China
CAT	-	Chloramphenicol Acetyltransferases
CDC	-	Centers for Disease Control and Prevention
CLSI	-	Clinical and Laboratory Standard Institute
dH_2O	-	Distilled Water
DHPS	-	Dihydropteroate Synthase
DNA	-	Deoxyribonucleic Acid
DO	-	Dissolved Oxygen
ESBL	-	Extended-spectrum Beta-lactamase
EtBr	-	Ethidium Bromide
EU	-	European Union
E-value	-	Expect value
FRI	-	Fisheries Research Institute
g	-	gram
GTP	-	Guanosine-5'-triphosphate
h	-	Hour/ Hours
kb	-	Kilo-base
kg	-	kilogram
LC-MS	-	Liquid Chromatography-Mass Spectrometry

MAR	-	Multiple Antibiotic Resistance
mg/L	-	Milligram/liter
MH	-	Mueller-Hinton
min	-	Minute/ Minutes
mL	-	Milliliter
MRA	-	Microbial Risk Assessment
MRSA	-	Methicillin-Resistant *Staphylococcus aureus*
mV	-	Millivolt
NA	-	Nutrient Agar
NARMS	-	National Antimicrobial Resistance Monitoring System
NASS	-	North American Spine Society
NB	-	Nutrient Broth
NCBI	-	National Center for Biotechnology Information
NTC	-	Non-template Control
NTU	-	Nephelometric Turbidity Unit
ORP	-	Oxidation Reduction Potential
PCR	-	Polymerase Chain Reaction
RIF	-	Rifampin
rRNA	-	Ribosomal Ribonucleic Acid
sec	-	Seconds
spp.	-	Species
TAE	-	Tris-acetate-EDTA
TDS	-	Total Dissolved Solids
UK	-	United-Kingdom
USA	-	United States
UV	-	Ultraviolet
V	-	Voltage
v/v	-	Volume per volume
VARSS	-	Veterinary Antimicrobial Resistance and Sales Surveillance
WHO	-	World Health Organization

LIST OF SYMBOLS

µ	-	Micro
β	-	Beta
+	-	Positive
−	-	Negative
σ	-	Shima
†	-	Dagger
°C	-	Degree Celcius
%	-	Percentage
>	-	Greater than

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CHAPTER 1

INTRODUCTION

1.1 Overview

Antibiotic resistance (ABR) refers to the intrinsic resistance of bacterial species towards certain antibiotics (Blair *et al.*, 2015). The bacteria develop the resistance gene which possessed the ability to tolerate the action of the antibiotic and hence led to the situation that antibiotics can no longer act as the ultimate way in the treatment of diseases or infections (Kathleen *et al*., 2016). Development of antibiotic resistance tends to cause threat to the human population along with other common bacterial infection that are difficult to be treated (Jindal *et al*., 2015).

The antibiotic resistance tends to emerge and spread among the bacterial population and also to the environment through the acquisition of resistance gene or by mutation (Sykes, 2010). Horizontal gene transfer is the crucial mechanism for the spreading of resistance gene among the environmental and the bacteria that share the same habitat and between phylogenetically closely related bacteria as well as induced by the stressors including the antibiotics (Bengtsson-Palme *et al.*, 2017). The fast-growing aquaculture sector have served as the reservoir in promoting the resistance mechanism due to the antibiotic that use in human medicine have been applied in the animal agriculture to promote growth and infection treatment (Done *et al*., 2015). Therefore, a crucial way of limiting the spread of antibiotic resistance is by reduce the usage of antibiotics to avoid the emergence of antibiotic resistance gene in the environment including aquaculture and aquatic sector (Cantón & Morosini, 2011).

1.2 Background of Study

Aquaculture sector generates important protein sources for the community. Diverse of bacterial species could be isolated from the aquaculture sources and its environment (Kathleen *et al.*, 2014). Previous studies showed that bacteria including the *Staphylococcus, Klebsiella, Pseudomonas, Aeromonas and Vibrio* could be found in the water and aquaculture sources including fish and shrimp (Akinbowale *et al.*, 2006). These bacteria could act as the important factor that causing diseases and infections in the aquaculture sources and eventually led to significant stock losses, which may contribute to lack of protein sources available and unable to meet with the demands from the market. It may also cause health problem of human as the human consumed the aquaculture sources. Hence, antibiotic agents including the antibiotics and antiviral have been widely used in various sectors including aquaculture to treat for the infections and for therapeutic purposes and it could be used as growth promoter in the aquaculture field as well to increase the feed efficiency of aquatic animal (Pelczar *et al.*, 1986). Commonly used antibiotics for agriculture and aquaculture including ampicillin, amoxycillin, cephalexin, tetracycline and erythromycin has been reported for the treatment of infections (Akinbowale *et al*., 2006). However, inappropriate overuse and misuse of the antibiotic agents has contributed to the development of antibiotic resistance genes among the microorganisms around the world. For bacteria, the resistance gene may widespread from one strain to another through horizontal gene transfer and result in the emerging threat to human population (Topp *et al.*, 2017). Therefore, ABR in pathogenic bacteria of aquatic animals could affect the disease management in the aquaculture systems and there are possibilities that the resistance determinants could be transferred to human pathogens from these systems (Sub-committee on Aquaculture, 2017).

1.3 Problem Statement

There are numerous of bacterial species present in the aquaculture sector and could cause from mild to severe infections to the aquaculture sources and the most

commonly used antibiotics including the ampicillin, amoxycillin, cephalexin, tetracycline and erythromycin has been reported for the treatment of infections. Epidemiological studies in Malaysia so far only focused on a single centre on emergence of certain antibiotics resistance bacteria in fish including the *Clarias gariepinus* and *Tilapia mossambica* (Budiati *et al*., 2013). Therefore, the emergence of antibiotic resistance bacteria especially in freshwater and the correlation between emergence of ABR in water and aquaculture sources are still poorly understood due to little serotyping data.

1.4 Research Objectives

This project was carried out to determine the antibiotic resistance bacteria of aquaculture sector in Johor, Malaysia. The aims and objectives of this project are:

I. To determine the water quality profile at each sampling sites.
II. To isolate the bacteria from aquaculture sources and water samples in Johor in term of antibiotics resistance.
III. To determine the MAR index value of antibiotics resistance bacteria (ARBs) in the aquaculture sources and water samples.
IV. To characterise the morphology of isolated bacteria with MAR index value more than 0.2 based on 16S rRNA gene sequencing analysis.

1.5 Significances and Original Contributions of Study

The findings of this study would contribute to more data on the antibiotic resistance bacteria (ARB) in the aquaculture sector and the pertaining data will be gathered via collaboration with Fisheries Research Institute (FRI), in which the gathered data will serve as input to integrate biological studies. In addition, this study would review the use of antibiotics and subsequent emergence of multiple antibiotic resistant bacterial species in aquaculture sector in Johor, Malaysia. Overcoming limitations on the existing literatures which did not thoroughly address the use of

antibiotics in aquaculture, this study could provide a better understanding on the emergence of antibiotic resistance bacteria.

1.6 Scope of Study

In this study, aquaculture source and water samples were collected from three sites in each location of Pulai, Sungai Melayu, Kukup, Pendas rivers and pond water at FRI. The samples collected were subjected to isolation of bacteria on nutrient agar and bacterial isolates were subjected to preliminary identification test including colony morphology and Gram Stain. Each isolate was subjected to antibiotic susceptibility test and the identified ARBs were proceed to polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) extraction. The PCR products were outsourced to Apical Scientific for 16S rRNA sequencing. After sequencing, analysis of the antibiotic resistance gene were carried out by BLASTn using publically available tools, such as National Center for Biotechnology Information (NCBI) and subject to phylogenetic analysis.

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