ANTIBIOTIC RESISTANCE PROFILE, POTENTIAL DRIVERS AND GENOTYPE OF *Escherichia coli, Salmonella* sp., AND *Vibrio* sp. ISOLATED FROM HYBRID RED TILAPIA AND ASIAN SEABASS IN WEST COAST OF PENINSULAR MALAYSIA

By

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

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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

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The intensive system of farm-raised fish accelerates the use of antibiotics which in turn increases the emergence of antimicrobial resistance (AMR) within bacteria in an aquatic environment. The current study was designed to investigate the prevalence of *E. coli, Salmonella* and *Vibrio sp.* among the cultured freshwater and brackish water fish in the central part of Peninsular Malaysia; determine the resistance pattern of *E. coli, Salmonella* and *Vibrio* isolates against selected antibiotics; identify aquaculture management practices and the environmental factors associated with the multidrug resistance (MDR); and characterize the isolates by molecular typing method to identify their profile and sequence type. Over a period of 12 months, a total of 32 fish farms from Selangor, Negeri Sembilan, Melaka and Perak were sampled. A total of 609 samples were collected from tilapia (312), Asian seabass (265) and pond waters (32). The samples were analyzed using established microbiology protocols to confirm *E. coli, Salmonella* sp, *Vibrios* sp. Disk diffusion and broth microdilution method (BMD), were used to determine the antibiotic resistance pattern of the isolates. Then, *V. parahaemolyticus* that harbored virulence genes were detected and characterized using Multi-Locus Sequence Typing (MLST) method. Risk factor for MDR *E. coli* (n=249) and *V. parahaemolyticus* (n=162) isolates were investigated applying multilevel binary logistic regression to observed the association of management and environmental factors to MDR development. The result of this study highlighted the aquaculture production system is an important reservoir of clinically important bacteria. The findings revealed that bacteria isolated had a high level of resistance to antibiotics classified as priority and critically important for human use, as well as veterinary critically important medications for food-producing animals, posing a significant risk to human, aquatic and animal health. Moreover, the virulent strain of *V. parahaemolyticus* found in this study are those that are circulating in the Asian geographical region from intercontinental dissemination. Possible interventions implementable at the aquaculture farm to reduce MDR *E. coli* include reducing on- farm practice such as manuring of fish pond. For *V. parahaemolyticus*, aquaculture farm adjacent to human activities, large-scale farms and farms with earthen ponds were at increasing odds for MDR development. This study findings propose potential practical and targeted interventions by aquaculture farmers to reduce AMR and mitigate development of MDR of significant bacteria of fish, animal and public health concern to better sustain the aquaculture industry while at the same time improve the welfare of animals and the health of environment and humans

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

PROFIL KERINTANGAN ANTIBIOTIK, PEMACU DAN GENOTIP *Escherichia coli, Salmonella* sp., *Vibrio* sp. BERASAL DARIPADA TILAPIA MERAH DAN SIAKAP DI BAHAGIAN PANTAI BARAT SEMENANJUNG MALAYSIA

Oleh

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Sistem intensif ternakan ikan mempercepatkan penggunaan antibiotik yang seterusnya meningkatkan kemunculan rintangan antimikrobial (AMR) bakteria dalam persekitaran akuatik. Kajian semasa telah direka untuk menyiasat prevalens *E. coli, Salmonella* dan *Vibrio* sp. pada ternakan ikan air tawar dan air payau di bahagian tengah Semenanjung Malaysia; menentukan corak rintangan pencilan E*. coli, Salmonella dan Vibrio* terhadap antibiotik terpilih; mengenal pasti amalan pengurusan akuakultur dan faktor persekitaran yang berkaitan dengan rintangan pelbagai ubat (MDR); dan mencirikan pencilan dengan kaedah taip molekul untuk mengenal pasti profil dan jenis jujukannya. Tiga puluh dua ladang akuakultur dari negeri Selangor, Negeri Sembilan, Melaka, dan Perak telah disampel dalam tempoh 12 bulan. Sebanyak 609 sampel telah dikumpulkan daripada tilapia (n=312), siakap (n=265) dan air kolam (n=32). Sampel dianalisis menggunakan protokol mikrobiologi yang tetap untuk mengesahkan pencilan *E. coli, Salmonella* sp., dan *Vibrio* sp. Kaedah diffusion disk dan Broth microdilution (BMD) digunakan untuk menentukan corak rintangan antibiotik bagi pencilan. *Vibrio parahaemolyticus* yang mengandungi gen virulensi diperiksa oleh penjenisan jujukan multi lokus (MLST). Faktor risiko MDR pada pencilan *E. coli* (n=249) dan *V. parahaemolyticus* (n=162) telah disiasat menggunakan multi pembolehubah berbilang peringkat. hasil kajian ini menekankan sistem pengeluaran akuakultur mungkin sebagai takungan bakteria penting secara klinikal di kawasan kajian. Penemuan mendedahkan bahawa pencilan mempunyai tahap rintangan yang tinggi terhadap antibiotik yang diklasifikasikan sebagai keutamaan dan sangat penting untuk kegunaan manusia serta haiwan penghasil makanan. Keadaan ini menimbulkan risiko besar kepada kesihatan manusia dan haiwan. Selain itu, strain virulen *V.*

parahaemolyticus yang ditemui dalam kajian ini adalah yang beredar di rantau geografi Asia dan antara benua. Intervensi yang mungkin boleh dilaksanakan di ladang akuakultur untuk mengurangkan MDR *E. coli* termasuk mengurangkan amalan pembajaan kolam ikan. Bagi *V. parahaemolyticus,* ladang akuakultur bersebelahan dengan aktiviti manusia, ladang berskala besar dan sistem kolam tanah berada pada peluang yang semakin meningkat untuk memperoleh MDR. Penemuan kajian ini mencadangkan intervensi praktikal yang boleh dilaksanakan oleh penternak akuakultur untuk mengurangkan AMR dan mengurangkan perkembangan MDR bakteria penting ikan, haiwan, dan manusia. Hasilnya untuk mengekalkan industri akuakultur dengan lebih baik dan meningkatkan kebajikan haiwan, kesihatan alam sekitar dan kesihatan manusia.

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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AMR	Antimicrobial resistance
Amp	Ampicillin
APW	Alkaline peptone water
ARGs	Antibiotic resistance genes
ATCC	American type culture collection
BGA	Brilliant green agar
BLAST	Basic local alignment search tool
BPW	Buffer peptone water
CDC	Centre for disease control and prevention
CI	Confidence interval
CLSI	Clinical and laboratory standards institute
Chl	Chloramphenicol
Cip	Ciprofloxacin
Cn	Gentamicin
Col	Colistin
Ctx	Cefotaxime
DAEC	diffusely adherent *E. coli*
DNA	Deoxyribonucleic Acid
DOF	Department of Fisheries
E	Erythromycin
EMB	Eosin methylene blue agar
Eft	Ceftiofur
EUCAST	European committee on antimicrobial susceptibility testing

ExPEC	Extrapathogenic *E. coli*
FAO	Food and agriculture organization of the United Nations
g	Gram
HGT	Horizontal gene transfer
IBM	International business machines
IMR	Institute of medical research
InPEC	Intrapathogenic *E. coli*
Is	Insertion sequences
iTol	Interactive Tree of Life
K	Kanamycin
LPS	lipopolisaccharide
µM	Micromolar
µL	Microliter
mg	Milligram
mL	Milliliter
MDR	Multi-drug resistance
MEGA	Molecular evolutionary genetics analysis
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
mRNA	Messenger ribonucleic acid
MS	Microsoft
Na	Nalidixic Acid
NA	Nutrient agar

NaCl	Natrium chloride
NJ	Neighbor joining
NTS	Non-typhoidal *Salmonella*
OIE	World Organization for animal health
OR	Odds Ratio
PBPs	Penicillin binding-protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
ppt	parts per thousand
rpm	Revolutions per minutes
R/I/S	Resistant/Intermediate/Sensitive
RVB	Rappaport Vassiliadis broth
S	Streptomycin
SEAs	Southeast Asian countries
SLVs	Single locus variants
SPSS	Statistical package for social sciences
ST	Sequence Type
STARS	Sequence Typing Analysis and Retrieval System
TBE	Tris-Borate ethylenediaminetetraacetic acid
TCBS	Thiosulfate-citrate-bile salts-sucrose agar
Te	Tetracycline
tRNA	Transfer ribonucleic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth

TLVs	Triple locus variants
W	Trimethoprim
WHO	World health organization
XLD	Xylose lysine deoxycholate

CHAPTER 1

INTRODUCTION

1.1 Study Background

The aquaculture sector has seen a rapid expansion worldwide owing to many reasons, viz. high demand for cheap and healthy protein compared to that obtained from livestock (cattle, sheep, goats), income generation, and employment opportunities, as well as its being a significant foreign exchange earner. Aquaculture represents one of the fastest-growing food-producing animal sectors over the last 25 years, surpassing the surge in livestock and dairy production substantially (Naylor, 2016). For instance, in 2008 alone, global aquaculture expanded significantly to 52.5 million tonnes worth about US$98.5 billion, constituting some 50% of the total food fish supply globally (Bostock et al., 2010). Recent study reported that global aquaculture production has more than tripled in live-weight volume from 34 Mt in 1997 to 112 Mt in 2017 (Naylor et al., 2021).

Elevated demand for fish has led to the rapid intensification of aquaculture in many countries, including those in Asia (Waite et al., 2014; Amal & Zamri-Saad, 2011;). Over the years, the intensification of aquaculture worldwide has led to outstanding growth and rapid expansion of the aquaculture industry. The semi-intensive culture (i.e. cage culture and concrete tank system) of tilapia fish farming system is practiced in Malaysia, Philippines, Thailand, and Indonesia (Gupta & Acosta, 2016). Unfortunately, such aquaculture production systems have been associated with some negative elements, including infectious diseases that adversely impact an otherwise successful system of aquaculture farming (Stentiford et al., 2020). The intensive fish culture system, along with high stocking density and poor husbandry practices, is strongly associated with stress and alterations in the environmental ecology of fish. This scenario creates an enabling epidemiological condition, facilitating the rapid spread of virulent strains of pathogens that may cause outbreaks of diseases in particular periods (Pulkkinen et al., 2010; Sundberg et al., 2016), altering immunological and metabolic robustness and triggering osmo-regulatory secondary stress responses (Vargas-Chacoff et al., 2014) that reduce the ability of the fish to fight an impending infection. Thus, infectious diseases represent significant constraints of an intensive aquaculture farming system (Sundberg et al., 2016). This has, accordingly, led to heavy usage of antimicrobials by aquaculture farmers, either for therapeutic purposes to cure bacterial infections or for prophylactic purposes to mitigate and curb the spread of infection in the fish population worldwide (Rigos et al., 2021; Miranda et al., 2018; Ranjan, 2017).

Along with the rapid expansion of the aquaculture industry, antibiotic consumption by South-east Asian countries has increased from 27 antibiotics used in aquaculture between 1990 and 2007 to 67 antibiotics in the period 2008 to 2018 (Lulijwa et al., 2020). The usage of antibiotics in the aquaculture led to the development of antimicrobial resistance (AMR) in aquaculture system (Cabello et al., 2016). For instance, a field study has revealed that the antimicrobial resistance genes (ARGs) conferring resistance to several antibiotics; tetracycline, sulfonamide, and trimethoprim bio-accummulated in the farm sediments of farms using antibiotics as compared to the other farms where the antibiotics were not used (Muziasari et al., 2016). Therefore, aquaculture represents a significant risk that can contribute to the emergence and dissemination of antimicrobial resistance and resistance genes posing significant public health threat due to its potential to disseminate AMR and ARGs to humans along the environment and the food chain (Larsson & Flach, 2021; Shen et al., 2019). Moreover, the direct exposure of the aquatic environment with extensive human activities and the continuity of water bodies linking different regions and continents of the globe, the spread and dissemination of pathogenic bacterial species harboring ARGs poses a global public health threat (Von Wintersdorff et al., 2016; Ding & He, 2010).

When compared to terrestrial animal farms, aquaculture is a complex, dynamic, linked system that is easily altered by environmental and anthropogenic variables (Zhao et al., 2020). Therefore, aquaculture is vulnerable to the introduction of antibiotic resistance bacteria and antibiotic resistance gene (Zhao et al., 2020) from the diverse source (Watts et al., 2017). For instance, the AMR of pathogens from human and animal agricultural settings including *Escherichia coli* and *Salmonella* spp. have been reported to be circulating in the Malaysian aquaculture systems (Sing et al., 2016; Ghaderpour et al., 2015; Budiati et al., 2013; Ng et al., 2014) and many other countries (Kagambèga et al., 2021; Liao et al., 2021; Ellis-Iversen et al., 2020; Kikomeko et al., 2016). Importantly, many resistant fish pathogens are zoonotic or potentially pathogens, that infect people both directly through contact with contaminated aquaculture facilities and indirectly through foodborne infections (Zhao et al., 2020). For instance, zoonotic aquatic pathogen such as *Vibrio parahaemolyticus* and *V. vulnificus* obtained from aquaculture products (Tan et al., 2020; Letchumanan et al., 2015b) and aquaculture farms in Malaysia (Amalina et al., 2019; Mohamad et al., 2019) were reported to resist a wide range of antimicrobials agent. Notwithstanding, there are few studies on the AMR and even fewer studies on AMR drivers in the aquaculture setting for important pathogens such as *E. coli, Salmonella*, *V. parahaemolyticus, V. vulnificus, V. cholerae* in Malaysia. In addition, the genetic diversity of these pathogens in the cultured fish in Malaysia is unknown. *E. coli* and *V. parahaemolyticus* are the target microorganisms to be investigated for AMR surveillance in Malaysia due to their importance to animal and public health (Noordin et al., 2020). Therefore, these bacteria will be the focus of the current study.

AMR has multiple drivers with aquaculture production and environment serving as an important element in the global epidemiology of AMR. Several studies such as by Zhang et al. (2020) and Borella et al. (2020) reported that resistance genes in the marine aquaculture are linked to terrestrial and anthropogenic sources. In addition, one of the AMR risk that is particularly pertinent to aquaculture is human and livestock/poultry waste that are applied to fertilize ponds in traditional integrated farming practices in shrimp industry (Thornber et al., 2020). Nevertheless, the impact of aquaculture farm practices and surrounding activities such as the local anthropogenic and agricultural activities, to the frequency of antibiotic resistance organism detection in Malaysia aquaculture has not been identified.

1.2 Problem Statement and Study Justification

Malaysia's aquaculture is considered as the fastest growing industry due to the increased of total production annually. For instance, the total production of aquaculture in 2019 has a significance surged about 5.19 % in quantity and 8.10% in value compared to 2018 (Department of Fisheries, 2019). The intensive aquaculture has promoted the bacterial diseases, which has led to the rise of antimicrobial usage in aquaculture and creating reservoirs of drug-resistance bacteria. Antimicrobial resistance is a global issue of tremendous veterinary and public health concern. Drivers for antimicrobial resistance include use of antibiotics in the human health, livestock husbandry and aquatic sectors. Unfortunately, very little information about the status of antimicrobial resistance in the aquatic animal especially those from the aquaculture farms in Malaysia. Aquaculture farm practices that may increase the risk of resistance among bacteria of public health concern is also unclear. In addition, the aquaculture environments may represent a channel of transmission for resistance to and from humans and other animals. Hospitals, human residence, livestock farms, and other agricultural activities have been correlated with the increasing antimicrobial selective pressures through their routine use of chemical and antibiotics. Nevertheless, the impact of changes in the activities surrounding farms such as the local anthropogenic and agricultural activities, to the frequency of antibiotic resistance detected has not been identified. In addition, the acceleration of multi-drug resistance development requires better identification of driving mechanism for its spread and transmission. In Malaysia, no studies have discerned the impact of these activities on the aquatic ecosystem and how they would impact the growing aquaculture industry. Therefore, the information is needed for the drivers of antimicrobial resistance in aquaculture animals to improve the aquaculture farm practices that may reduce the risk of resistance among bacteria of public health concern. Furthermore, there is still the gap knowledge regarding occurrence of public health significance bacteria in aquaculture system and its genetic diversity. This information is essential to provide an understanding in epidemiology and public health intervention development of AMR in public health concern bacteria in the aquaculture settings.

1.3 Research Questions

i. What are the prevalence of *E. coli, Salmonella* & *Vibrio* among cultured tilapia and Asian seabass in the west coast of Peninsular Malaysia?

ii. What are the antibiotic resistance profiles/susceptibility pattern of *E. coli, Salmonella* & *Vibrio* isolates?

iii. What are the epidemiological risk factors that contribute to the occurrence of multidrug resistance *E. coli, Salmonella* & *Vibrio* among cultured tilapia and Asian seabass in the west coast of Peninsular Malaysia?

iv. What are the genotypes of virulence genes associated *Vibrio parahaemolyticus*?

1.4 Research Hypothesis

i. The prevalence of *E. coli, Salmonella* & *Vibrio* among cultured tilapia and asian seabass fish in the west coast of Peninsular Malaysia are high

ii. *E. coli, Salmonella* & *Vibrio* isolates from cultured tilapia and asian seabass fish are multidrug resistant

iii. Certain aquacuture farm management, as well as anthropogenic activities surrounding farms factors play a role in the occurrence epidemiology of multidrug resistance among public health concern bacteria.

iv. The virulence genes associated *V. parahaemolyticus* recovered from Asian seabass fishes are genetically diverse.

1.5 Objectives

The objectives of this study are :

i. To estimate the prevalence of *E. coli, Salmonella* & *Vibrio* in cultured tilapia & Asian seabass fish as well as its environment in the west coast of Peninsular Malaysia

ii. To describe the AMR pattern of *E. coli, Salmonella* & *Vibrio* and its epidemiological risk factor associated with multi-drug resistance occurrence in cultured tilapia & Asian seabass fish in the west coast of Peninsular Malaysia.

iii. To determine the genetic diversity of *V. parahaemolyticus* isolated from cultured Asian seabass in the west coast of Peninsular Malaysia.

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