UNIVERSITI PUTRA MALAYSIA

ISOLATION, CHARACTERIZATION, AND PATHOGENICITY OF NONAND MYO-INOSITOL UTILIZING Aeromonas *SPECIES ISOLATED FROM CULTURED FRESHWATER FISHES IN PENINSULAR MALAYSIA*

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By

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ISOLATION, CHARACTERIZATION, AND PATHOGENICITY OF NON- AND MYO-INOSITOL UTILIZING A*eromonas* SPECIES ISOLATED FROM CULTURED FRESHWATER FISHES IN PENINSULAR MALAYSIA

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Chair : Assoc. Prof. Ina Salwany Md Yasin PhD
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Aeromonas spp. are ubiquitous in water bodies ranging from environmental water to aquaculture water. This genus poses a threat to a myriad of hosts, including freshwater fishes. High stocking density and improper farm practices can increase the susceptibility of cultured freshwater fishes to diseases. Motile *Aeromonas* septicemia (MAS) is a bacterial disease that is caused by *Aeromonas* spp. and this disease has infected many freshwater fishes, causing huge economic losses to some countries. Furthermore, many studies have reported the presence of multiple virulence genes and antibiotic resistance abilities among these notorious *Aeromonas* spp. In addition, with the recent reports of the emergence of a hypervirulent pathotype of *A. hydrophil*a (vAh) that can cause persistent MAS, are particularly of increasing concern among researchers and fish farmers. The vAh strain can be distinguished from other non-vAh strain by its ability to utilize *myo*-inositol as a sole carbon source. However, the contribution of *myo*-inositol to the virulence of *Aeromonas* is poorly understood and never recorded in Malaysia. This data on *myo*-inositol utilizing *Aeromonas* spp., including vAh, could alarm Malaysia's freshwater aquaculture on the approaching danger. Therefore, the objectives of this study were to characterize *Aeromonas* spp. from freshwater fish farms in Malaysia and analyze the isolates for the presence of putative virulence genes and antibiotic resistance abilities, to identify *myo*-inositol utilizing strain among isolated *Aeromona*s spp. and elucidate the influence of *myo*-inositol utilizing ability on their virulence in red hybrid tilapia, and to perform whole-genome sequencing of *myo*-inositol utilizing *Aeromonas* spp. Briefly, a total of 124 isolated *Aeromonas* spp. were collected and screened for putative virulence genes, antibiotic resistance properties, and *myo*-inositol utilizing ability, from nine fish farms, located in various geographical regions in Peninsular Malaysia. The results revealed that, out of 124 *Aeromonas* isolates collected, five species of *Aeromonas* spp., including *A. dhakensis, A. hydrophila, A. veronii, A. caviae* and *A. jandaei,* with multiple virulence genes and antibiotic resistance abilities were characterized. Furthermore, only strains of *A. dhakensis* (2%) and *A. hydrophila* (20%) were found to have all eight virulence genes studied, and 69% of all *Aeromonas* spp. have MAR index of more than 0.2. Only one *myo*-inositol utilizing strain, *A. dhakensis* 1P11S3 was identified, among 124 *Aeromonas* spp. Based on its virulence gene assessment, *myo*-inositol utilizing *A. dhakensis* 1P11S3 only harbored 5 of the 8 virulence genes tested in this study. In addition, the antibiotic disc susceptibility test revealed that *myo*-inositol utilizing *A. dhakensis* 1P11S3 was only resistant to less than 40% of the antibiotics tested. Further analysis was done via an experimental challenge against red hybrid tilapia (*Oreochromis* sp.) using selected non- and *myo*-inositol utilizing *Aeromonas* spp. (n = 6). The $LD_{50\text{-}240h}$ result revealed that the lowest were *A. dhakensis* 4PS2 and *A. hydrophila* 8TK3 (10^5 CFU/mL), followed by *myo*-inositol utilizing *A. dhakensis* 1P11S3 (10^7 CFU/mL), *A. veronii* 6TS5 (10^7 CFU/mL), *A. caviae* 7X11 (10^7 CFU/mL) and the highest was *A. jandaei* 7KL3 (10^{11} CFU/mL). Additionally, no significant difference in histopathological changes of the kidney, liver, and spleen, caused by *myo*-inositol utilizing *A. dhakensis* 1P11S3, as compared to other non-*myo*-inositol utilizing *Aeromonas* following experimental challenge in the red hybrid tilapia. Whole-genome sequencing of *A. dhakensis* 1P11S3 revealed that *myo*-inositol utilizing *A. dhakensis* 1P11S3 was indeed *A. dhakensis,* which was the first record of *A. dhakensis* that utilize *myo*-inositol as a sole carbon source. Further *in silico* analysis with other *A. hydrophila* revealed that they are closely related and inositol catabolism was confirmed to be present in genotypes of *A. dhakensis* 1P11S3. In conclusion, *myo*-inositol utilizing ability did not cause significantly increased pathogenicity in *A. dhakensis* with current disease model. It may be due to different genomic representation of *myo*-inositol utilizing vAh with *A. dhakensis* 1P11S3, since both are of different *Aeromonas* spp. Furthermore, there is a possibility of other virulence factors that are exclusive to only vAh. Nevertheless, horizontal gene transfer may have occurred between the vAh strain and *A. dhakensis* 1P11S3, which to the author’s knowledge, no study has reported this ability among *A. dhakensis.* Future studies should be done to knockout the *myo*-inositol gene cluster from *A. dhakensis* 1P11S3 and compare its pathogenicity with wild type *A. dhakensis,* which can further elucidate the influence of *myo*-inositol gene cluster on the virulence of *Aeromonas* spp. Nonetheless, findings from this study would provide a recent epidemiological data on *Aeromonas* spp., that would help in the development of vaccine and therapeutants for a sustainable freshwater aquaculture.

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

ISOLASI, KARAKTERISASI, DAN PATOGENISITI PENGGUNA DAN BUKAN PEGGUNA MYO-INOSITOL DALAM KALANGAN SPESIS *Aeromonas* DIDAPATI DARI IKAN AIR TAWAR DI SEMENANJUNG MALAYSIA

Oleh

MOHAMAD AZZAM BIN MOHD SAYUTI

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Spesis Aeromonas berada di mana-mana, bermula dari persekitaran air sehinggalah air di aktiviti akuakultur. Genus ini mempunyai pelbagai sasaran perumah, termasuklah ikan air tawar. Ketumpatan stok yang tinggi dan praktis yang tidak sepatutnya, boleh mengakibatkan peningkatan risiko jangkitan kepada penternakan ikan air tawar dan salah satu penyakit utama dalam akuakultur adalah “Motile Aeromonas Septicemia” (MAS). Spesis Aeromonas adalah punca penyakit MAS dan penyakit ini telah mengakibatkan kerugian yang besar kepada beberapa buah negara. Tambahan pula, terdapat banyak kajian yang melaporkan kewujudan spesis Aeromonas dengan beberapa gen virulen dan kebolehan ketahanan antibiotik. Di samping itu, dengan adanya patogen yang sangat berbahaya, vAh, iaitu salah satu patogen A. hydrophila, telah merisaukan para penternak ikan dan juga penyelidik. vAh boleh dikenalpasti daripada spesis Aeromonas yang lain melalui kebolehannya mengguna myo-inositol sebagai punca karbon. Walau bagaimanapun, sehingga kini pengaruh kebolehan ini kepada jangkitan Aeromonas masih kurang difahami dan tidak pernah direkodkan di Malaysia. Data melibatkan spesis Aeromonas yang boleh menggunakan myo-inositol, termasuk vAh, boleh memberi amaran tentang bahaya kepada industri akuakultur air tawar di Malaysia. Oleh itu, projek ini bertujuan i) untuk mengenal pasti spesis Aeromonas dalam ternakan ikan air tawar di Malaysia dan menganalisis keuwujudan gen virulen dan kebolehan ketahanan antibiotik antara mereka, ii) untuk mengenal pasti isolat positif myo-inositol dalam kalangan spesis Aeromonas dan menerangkan pengaruh kebolehan ini kepada jangkitan spesis Aeromonas, dan iii) untuk menganalisis keseluruhan genom isolat positif myo-inositol dalam kalangan spesis Aeromonas. Eksperimen dimulai dengan proses isolasi dan analisis kewujudan gen virulen, tahap ketahanan antibiotik, dan kebolehan menggunakan myo-inositol, dalam kalangan spesis Aeromonas (n = 124) dari sembilan buah ladang penternakan ikan air tawar di Semenanjung Malaysia. Kemudian, seluruh isolat (n = 124) diujikaji untuk mencari isolat positif myo-inositol. Keputusan menunjukkan lima spesis Aeromonas seperti A. dhakensis, A. hydrophila, A. veronii, A. caviae dan A. jandaei, yang mempunyai pelbagai gen virulen dan ketahanan antibiotik telah dikenalpasti. Di samping itu, hanya spesis A. dhakensis (2%) dan A. hydrophila (20%) mempunyai kesemua lapan gen virulen. Selain itu, sebanyak 69% spesis Aeromonas mempunyai indeks MAR melebihi 0.2. Tambahan pula, hanya satu isolat positif myo-inositol telah dikenalpasti daripada kesemua 124 spesis Aeromonas. Kemudian, isolat positif myo-inositol ini mempunyai hanya lima daripada lapan kewujudan gen virulen dan hanya mempunyai ketahanan kepada 40% antibiotik digunakan. Seterusnya, eksperimentasi jangkitan dalam tilapia merah dijalankan bagi isolat positif dan negatif myo-inositol. Eksperimentasi dalam tilapia kacukan merah menunjukkan dos mematikan median (LD50-240h) adalah terendah bagi A. dhakensis 4PS2 and A. hydrophila 8TK3 (105 CFU/mL), diikuti oleh isolat positif myo-inositol A. dhakensis 1P11S3 (107 CFU/mL), A. veronii 6TS5 (107 CFU/mL), A. caviae 7X11 (107 CFU/mL) dan tertinggi adalah A. jandaei 7KL3 (1011 CFU/mL). Tambahan pula, tiada perbezaan histopatologi dalam buah pinggan, hati dan limpa, yang signifikan ditunjukkan oleh isolat positif myo-inositol ini daripada isolat Aeromonas yang lain. Akhir sekali, eksperimen dijalankan untuk mengenalpasti genom lengkap isolat positif myo-inositol, A. dhakensis 1P11S3. Hasilnya, genom lengkap A. dhakensis 1P11S3 dapat dihasilkan dan ia terbukti adalah dari spesis A. dhakensis, dan spesis ini mempunyai hubungan rapat dengan spesis A. hydrophila. Di samping itu, kebolehan mengguna myo-inositol adalah tepat berdasarkan data dari genom lengkap A. dhakensis 1P11S3. Konklusinya, kebolehan mengguna myo-inositol tidaklah membantu kepada jangkitan A. dhakensis dalam projek ini dan lebih banyak data diperlukan untuk membuktikan hipotesis ini. Hal ini disebabkan mungkin kerana vAh dan isolat positif myo-inositol (A. dhakensis 1P11S3) mempunyai genetik yang berbeza. Tambahan pula, ada kemungkinan kewujudan faktor virulensi yang hanya terdapat di vAh. Walaubagaimanapun, terdapat kemungkinan telah berlakunya pergerakan gen mendatar di antara vAh dan A. dhakensis 1P11S3, kerana kebolehan ini tidak pernah dilaporkan sebelum ini. Eksperimen akan datang perlu fokus kepada membuang kluster gen myo-inositol dalam isolat tersebut, dan bandingkan kebolehjangkitan isolat terbabit dengan isolat A. dhakensis yang lain, supaya dapat dirungkaikan dengan lebih jelas pengaruh kebolehan menggunakan myo-inositol terhadap kebolehjangkitan dalam spesis Aeromonas. Data daripada ekpserimen ini, dapat memberikan maklumat terbaru tentang epidemiologi spesis Aeromonas, disamping membantu dalam proses penghasilan vaksin demi kebaikan akuakultur air tawar.

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LIST OF ABBREVIATIONS

MAS	motile *Aeromonas* septicemia
vAh	hypervirulent strain of *A. hydrophila*
HGT	horizontal gene transfer
UPM	Universiti Putra Malaysia
^{o}C	degree celsius
LDCs	least developed countries
LD$_{50}$	median lethal dose
GC-FAME	fatty acid methyl ester
MALDI-TOF MS	matrix-assisted laser desorption ionization mass spectrometry-time of flight
ICSP	International Committee of Systemics of Prokaryotes
PCR	polymerase chain reaction
ERIC-PCR	enterobacterial repetitive intergenic consensus-PCR
AFLP	amplified fragment length polymorphism
PFGE	pulsed-field gel electrophoresis
MLST	multilocus sequence typing
Ast	cytotoxic enterotoxins
TNF-α	tumor necrosis factor α
α	alpha
β	beta
γ	gamma
IL-1β	interleukin
T3SS	type three secretion system
T2SS	type two secretion system
T5SS	type five secretion system

AJC	apical junction complex
ROS	reactive oxygen species
NO	nitric oxide radicals
DO	dissolved oxygen
BOD	biological oxygen demand
COD	chemical oxygen demand
TDS	total dissolved solid
TSS	total suspended solids
ASA	*Aeromonas* selective agar-base
TSB	tryptic soy broth
ahp	serine protease gene
fla	flagella gene
act	cytotoxic enterotoxin gene
ela	elastase gene
hly	hemolysin
alt	heat-labile cytotonic enterotoxin
lip	lipase gene
aer	aerolysin gene
MHA	Mueller-Hinton agar
MAR	multiple antibiotic resistance
AK	amikacin
AMP	ampicillin
CTX	cefotaxime
LEV	levofloxacin
AMC	amoxycillin

CN	gentamicin
C	chloramphenicol
TET	tetracycline
CFU/mL	colony forming units per millilitre
mL	millilitre
g	gram
kg	kilogram
SD	standard deviation
bplA	probable oxidoreductase gene
hitC	iron (III) ABC transporter gene
T6SS	type VI secretion system
PF	pass filter
tRNAs	transfer RNAs
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
COG	Cluster of Orthologous Groups
ANI	average nucleotide identities
ncRNA	non-coding RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
GT	glycosyl transferase
GH	glycoside hydrolases
AA	auxiliary activities
PL	polysaccharide lyases
CBM	carbohydrate-binding molecules
CE	carbohydrate esterases

ORF	open reading frame

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Fish is becoming a more important food source in the whole world. Global fish production, including aquaculture and fishery, had increased from 142.5 million tonnes in 2008 (USD 192 billion) to 178.5 million tonnes (valued at USD 401 billion) in 2018 (FAO, 2010; FAO, 2020). Despite the fact that aquaculture has grown dramatically over the last three decades, infectious disease has proven a stumbling block to further development in the aquaculture sector. Aquaculture often necessitates large-scale production facilities and high-density animal populations, which create stress among cultured fish, increasing the risk of infection (Bebak et al., 2015; Zdanowicz et al., 2020). .

One of the most important diseases in aquaculture is motile *Aeromonas* septicemia (MAS). Motile *Aeromonas* septicemia is caused by the species of *Aeromonas* such as *A. hydrophila, A. dhakensis* and *A. veronii* (Singh et al., 2008; Kozińska & Pkala, 2012; Jagoda et al., 2014; Dong et al., 2017; Amal et al., 2018; Basri et al., 2020). Bacteria of the genus *Aeromonas* are Gram-negative, typically opportunistic bacterial pathogens that are ubiquitous in various aquatic bodies such as freshwater environments (Tomás, 2012). Even though *Aeromonas* spp. such as *A. hydrophila* is notoriously known as a pathogen of freshwater cultured fishes, its occurrence and health impacts are usually stress-related and is secondary to infection by a primary pathogen (Peatman et al., 2018). Pathogenesis of *Aeromonas* spp. in target host was multifactorial since there are many virulence factors produced by the members of this genus.The combination of virulence factors such as hemolysin and aerolysin secreted by *A. hydrophila,* have been linked to multiple hemorrhages found on the external body of Nile tilapia (*Oreochromis niloticus*) (Omar et al., 2016; Jung-Schroers et al., 2019).

Treatment of fish clinically infected with *Aeromonas* spp. is possible by applying antimicrobial substances. However, due to frequent and excessive use of antibiotics by fish farmers, as well as other prophylactic substances, they caused the development of drug-resistant pathogen (Pauzi et al., 2020). The increasing incidence of multidrug resistance amongst *Aeromonas* spp., have been reported in previous studies such as imipenem, tetracycline, erythromycin, kanamycin and ciprofloxacin (Chen et al., 2014; Chandrarathna et al., 2018). In order to prevent the increasing bacterial resistance cases among *Aeromonas* spp., the administration of antibiotics needs to be appropriately regulated and handled.

Motile *Aeromonas* septicemia was not a significant concern for the catfish industry in the United States until 2009, a hypervirulent strain of A. *hydrophila* (vAh) had emerged. The strain was found in the Alabama and east Mississippi catfish farming regions, causing severe economic losses due to rapid death of 10-100% mortality among channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* × *I. furcatus*) within one week (Hossain et al., 2013; Peatman et al., 2018). Upon examination, fish exhibited typical symptoms of MAS, but lacked of other concurrent infections (Baumgartner et al., 2017). This outbreak by vAh was so severe that it was estimated 8 million of fish death was the lost suffered in 2010, due to mass mortalities of food-sized catfish in Alabama (Hossain et al., 2013). Recently, the number has increased to 30 million of losses in Alabama (Barger et al., 2021). The impact the vAh isolate can also be seen in China's Guangdong Province where it was reported that the loss was approximately five billion yuan per year (Rasmussen-Ivey et al., 2016). Even though data on production loss solely attributed to epidemics of vAh is hard to obtain, it cannot be denied that aquaculture worldwide has significantly been impacted by vAh (Hossain et al., 2013; Zhang et al., 2014).

Although few studies have reported the virulence of vAh, the specific virulence factors that significantly contribute to the hypervirulence properties of vAh are poorly understood. However, all vAh strains share common ability to utilize *myo*-inositol as a sole carbon source. This unique *myo*-inositol metabolism in vAh may have been linked to its virulence, and this was reported by Hossain et al. (2013), where it was found that *myo*-inositol metabolism gene cluster was situated in epidemic-associated genomic island of all vAh strains. In addition, none of the *Aeromonas* spp., aside from vAh of *A. hydrophila*, is known to utilize *myo*-inositol as a sole carbon source.

Acquisition and loss of foreign genetic elements among bacterial pathogens is always the main culprit of the emergence of infectious pathogens (Rasko et al., 2011). This phenomenon was driven by horizontal gene transfer (HGT), where genetic elements were transferred via prophages, integrating conjugative elements and plasmids, which sometimes resulted in heightened virulence of pathogen (Hossain et al., 2013). The rapid cases of epidemics of vAh suggested that HGT may have been directly involved (Hossain et al., 2013). Through genomic comparison, analysis on vAh strains isolated from catfish in the USA and carp in China, indicated these strains share a recent common ancestor and USA vAh may have potential Asian origin (Hossain et al., 2014; Rasmussen-Ivey et al., 2016; Hossain et al., 2016; Tekedar et al., 2019).

1.2 Problem statements

There have been several previous reports on *Aeromonas* spp. infection in cultured fishes in Malaysia (Musa & Laith, 2014; Hamid et al., 2016; Amal et al., 2018; Pauzi et al., 2020), but no study that has comprehensively described the distribution of *Aeromonas* spp. in the Malaysian freshwater aquaculture. Therefore, the epidemiology of aeromoniasis in Malaysia's aquaculture remains poorly understood. In addition, due to the fact that members of this genus are closely related (Soler et al., 2004), there is a possibility of misidentification cases happened in past literatures (Aravena-Román et al., 2011). Furthermore, there are limited data on the putative virulence genes and antibiotic resistance profiles of *Aeromonas* spp. in Malaysia.

The recent emergence of a hypervirulent pathotype of *A. hydrophila* (vAh strain), poses a threat to global aquaculture production and have been reported in USA and China (Bebak et al., 2015; Pang et al., 2015; Rasmussen-Ivey et al., 2016). However, limited information is available as to how far the pathogen has traveled around the globe, and there is a possibility that the vAh is already present in Malaysia. Previous report on the pathogenicity of vAh determined that the strain was very virulent, as compared to typical *A. hydrophila* (Hossain et al., 2013; Zhang et al., 2016). However, all these data on the pathogenicity of vAh are still considered few, and only limited hosts were tested. In addition, no study has reported other *myo*-inositol utilizing *Aeromonas* spp., aside from vAh.

Lastly, since no study has been done on vAh or other *myo*-inositol utilizing *Aeromonas* spp., the whole-genome sequences of the *Aeromonas* spp. strains from Malaysia are still not yet identified.

1.3 Significance of the study

This study will provide an up-to-date extensive data on the prevalence of *Aeromonas* spp., as well as characteristic of *myo*-inositol utilizing *Aeromonas* spp., including vAh in different species of cultured freshwater fish in Peninsular Malaysia. Moreover, the current state of the putative virulence genes and antibiotic-resistance profiles of *Aeromonas* spp. can be reported. Data from this study can contribute to the fish health management plan, such as National Fish Health Strategy Malaysia 2018–2022, which encouraged the latest data on antimicrobial resistance patterns alongside with presence of virulence genes among *Aeromonas* spp. in Peninsular Malaysia.

Since this study will potentially reveal the prevalence of vAh, further experimental challenge in live host will help researchers to elucidate the influence of *myo*-inositol-utilizing ability on the virulence of *Aeromonas* spp. In addition, the draft genomes of *myo*-inositol utilizing *Aeromonas* spp., will be useful for the understanding of the source of HGT and discovery of novel putative virulence gene clusters in the future study.

1.4 Objectives of the study

This study in general, embarks on the following specific objectives:

i. To determine the prevalence of *Aeromonas* spp. in cultured freshwater fishes that collected from various geographical region in Peninsular Malaysia, along with identification of multiple putative virulence genes and antibiotic resistance properties.

ii. To screen and examine the influence of *myo*-inositol utilizing ability on the virulency of *Aeromonas* spp. in experimental infection of red hybrid tilapia (*Oreochromis* sp.) as an animal model.

iii. To analyze the first whole-genome sequence of *myo*-inositol utilizing *Aeromonas* spp. strain that was isolated from diseased cultured fish in Malaysia.

1.5 Hypotheses of the study

1. Objective 1.
   H_0: There are no difference in the prevalence, presence of putative virulence genes and antibiotic resistance profiles of *Aeromonas* spp. in nine different fish farms in Peninsular Malaysia
   H_α: There are difference in the prevalence, presence of putative virulence genes and antibiotic resistance profiles of *Aeromonas* spp. in nine different fish farms in Peninsular Malaysia

2. Objective 2.
   H_0: There are no difference in the histopathological changes caused by non- and *myo*-inositol utilizing *Aeromonas* spp. in red hybrid tilapia
   H_α: There are difference in the histopathological changes caused by non- and *myo*-inositol utilizing *Aeromonas* spp. in red hybrid tilapia different fish farms in Peninsular Malaysia

3. Objective 3.
   H_0: There is no complete *myo*-inositol metabolism found in the genomes of *myo*-inositol utilizing *Aeromonas* spp.
   H_α: There is a complete *myo*-inositol metabolism found in the genomes of *myo*-inositol utilizing *Aeromonas* spp.

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