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CHARACTERIZATION AND PATHOGENICITY OF Aeromonas hydrophila ISOLATED FROM FRESHWATER FISHES

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By

RUHIL HAYATI HAMDAN

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

April 2017

DEDICATION

Dedicated to my late mother...

Habesah Ismail

(12.10.1952 – 10.10.2016)

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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April 2017

Chairman : Hassan Hj Mohd Daud, PhD
Faculty : Veterinary Medicine

Aeromonas hydrophila is ubiquitous in aquatic habitat but frequently causes ulcerative disease known as red sore disease, red rot disease and motile Aeromonas septicemia (MAS) disease in cultured and feral fish. The disease is common worldwide and resulted in million dollar economic losses in the freshwater fish farming industry. Thus, this study was carried out to identify morphological, biochemical and physiological characteristics of *A. hydrophila* isolated from clinically infected freshwater fish in the state of Selangor, Malaysia. All sampled fish showed non-specific clinical signs such as skin hemorrhages, sunken eyes and fin rot. Forty isolates of *A. hydrophila* were isolated from 150 wild and farmed fishes by direct streakings from skin, kidney, spleen and liver and overlaid onto trypticase soy agar. Species of fish sampled were Black tilapia, *Oreochromis mossambicus* (n=30); Red hybrid tilapia, *Oreochromis* sp. (n=30); Jade perch, *Scortum barcoo* (n=25); Javanese carp, *Puntius gonionotus* (n=10); River carp *Leptobarbus hoevenii* (n=10), River catfish, *Pangasius pangasius* (n=10); Climbing perch, *Anabas testudineus* (n=10); African catfish, *Clarias gariepinus* (n=20) and Louhan, *Cichlasoma* sp. (n=5). All isolates were identified using commercial kit, API 20E in combination with Gram staining, oxidase, catalase, vibriostatic agent, NaCl tolerance, temperature tolerence and hemolytic activity. Morphologically, all isolates were short rods, Gram negative and motile. The biochemical tests showed positive results for oxidase, catalase, ONPG, ADH, LDC, citrate utilization, indole production, gelatinase and Voges Proskauer. Besides, negative results were obtained from ODC, H_2S, urease, TDA. Acid was produced from glucose, mannitol and arabinose. No acid production from inositol, sorbitol, rhamnose, saccharose, melibiose and amygdalin. All isolates grew in 0% and 3% NaCl. The bacterial isolates also grew at 22ºC and 37ºC. All isolates showed β-hemolysis on horse blood agar. Furthermore, *A. hydrophila* isolates were further confirmed using 16S rDNA sequencing.

In the present study, the presence of virulence genes in 40 isolates of *A. hydrophila* obtained from freshwater fishes was carried out using polymerase chain reaction

(PCR). The ten virulence genes were cytotonic heat-labile (*alt*), cytotonic heat-stable enterotoxins (*ast*), cytotoxic heat-labile enterotoxin (*act*), hemolysin (*hly*), aerolysin (*aer*), flagella A and flagella B (*fla*), lipase (*lip*), elastase (*ela*), serine protease (*ser*), and DNases (*exu*). All isolates contain *lip*, *exu* and *ser* genes. But, none of them contain *ela*, *alt* and *laf* genes. Almost all the isolates have *hly* (95%) and *aer* (75%) genes. 30% of the *A. hydrophila* isolates showed the presence of *ast* and *act* genes. Twelve out of 40 isolates have the highest genes content (*lip*+, *exu*+, *ast*+, *act*+, *hly*+, *aer*+, *ser*+) while, two isolates have the lowest genes combination (*lip*+, *exu*+, *ser*+).

Aeromonas hydrophila isolated from Louhan fish (AHFH40) which contained high combination of virulence genes (lip^+, exu^+, ast^+, act^+, hly^+, aer^+, ser^+) was chosen for experimental infection in *Oreochromis* sp. Healthy juveniles of *Oreochromis* sp. (average size of 4 inches) were injected intraperitoneally with 0.1 ml of *A. hydrophila* suspension containing 10^3, 10^4, 10^5, 10^6,10^7 and 10^8 cfu/ml. Susceptibility to experimental infection was expressed as LD_{50} calculated by the method of Reed and Muench (1938). Haematological and biochemistry parameters were obtained from the control and experimentally infected fish at day 1, 3, 5 and 7. Significant reductions were observed in the hemoglobin content at 7 days post inoculation with *A. hydrophila*. The mean values of RBC, PCV and Hb showed fluctuation, but they were significantly lower (P<0.05) than those of the controls at 3, 5 and 7 dpi. Compared to the control group, the infected fish showed decreased in MCHC and MCH mean values at 5 and 7dpi. The WBC mean values of infected fish showed higher significant changes at 5 and 7dpi, respectively. Mean biochemical indices of TP, ALT and AST levels also fluctuated, and there were significant differences (P<0.05) between the infected and control groups. The glucose levels in infected fish were initially increased significantly (P<0.05) from the control mean values. Fishes showed clinical signs of MAS were sacrificed for histopathological assessment. Tissues from kidney, liver and spleen were fixed in 10% buffered formalin, processed and embedded into paraffin wax blocks. The sections were cut at 3-4 μm thick and stained with haematoxylin and eosin. Current results showed the LD_{50} of *A. hydrophila* to *Oreochromis* sp. was calculated ranging between 10^4 and 10^5 cfu/ml. Histopathological changes which included mononuclear cell infiltration, necrosis and hemorrhages in the kidney, liver, spleen and hepatopancreas were postulated due to the cytotoxin produced by the bacteria.

Additionally, antibiotic sensitivity test, plasmid profiling and integron detection of *Aeromonas hydrophila* were also carried out. The isolates were tested for sensitivity to 10 antibiotics namely, penicillin G, cephalexin, florfenicol, streptomycin, kanamycin, erythromycin, ampicilin, gentamicin, oxytetracycline and tetracycline. Then, Multiple Antibiotic Resistance (MAR) index was calculated to determine the usage of antibiotics by fish farmers. Besides, polymerase chain reaction was carried out to detect integrase genes *Int1*, *Int2* and *Int3*, gene cassette, integron-associated *aadA*, *sul1* and *qac1* genes, streptomycin resistance genes *strA-strB*, β-lactamase resistance genes bla_{TEM} and bla_{SHV}, and tetracycline resistance genes *tetA-E* and *tetM.* In the present study, *A. hydrophila* was shown to be sensitive to erythromycin, florfenicol, kanamycin and oxytetracycline, while they were resistance to cephalotin, gentamicin and penicillin-G. Furthermore, Multiple Antibiotic Resistance (MAR) index for the bacterial isolates was ranging from 0.4 to 0.5. The current MAR results indicated that the *A. hydrophila* in these farmed fish might have been indiscriminately and continuously exposed to those

antibiotics during the fish culturing stages. Twelve out of 40 isolates contained plasmid DNA bands with sizes ranging from 6 to 23 kb. There was a little variation in the plasmid sizes observed. In addition, no particular plasmid profiles were predictive of a particular pattern of antibiotic resistance. The *intl*1 gene was detected in 50% (20/40) of *A. hydrophila* strains but no isolates contain *intl*2 and *intl*3. No gene cassette was detected from all the *A. hydrophila* isolates. The *aadA* was found in 5/40 (12.5%) of *A. hydrophila* isolates, while both of tetracycline resistance genes, *tet*A and *tet*C were found in 16/40 (40%) of the isolates. The *strA-strB*, bla_{TEM} and bla_{SHV} genes were not detected in any of the isolates. This present study was the first report of integron detection in *A. hydrophila* isolates in Malaysia.

The findings obtained in this study indicated that *A. hydrophila* infection has become an important health issue in fish farms. Likewise, the increase of antibiotics resistant Aeromonads will lead to treatment challenge in *Aeromonas* infection. This study demonstrated that the presence of different combinations of the virulence genes in *A. hydrophila* isolates indicating their probable roles in the pathogenesis of *Aeromonas* infections. Results from haematological and histopathological studies provided valuable information on the extensity of pathology associated with Aeromoniasis in *Oreochromis* sp. Constant monitoring should be done in order to compile more information on antibiotic sensitivity of *Aeromonas* spp. and other known aquatic bacteria species in order to avoid the development of antibiotic superbug. The detection of resistance determinant genes in *A. hydrophila* is of public importance and environment significance.

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

PENCIRIAN DAN PATOGENISITI *Aeromonas hydrophila* YANG DIPENCILKAN DARIPADA IKAN AIR TAWAR

Oleh

RUHIL HAYATI HAMDAN

April 2017

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Aeromonas hydrophila sentiasa berada di dalam habitat akuatik dan sering menyebabkan penyakit ulser iaitu penyakit kudis merah, penyakit karat merah and septisemia *Aeromonas* motil (MAS) pada ikan kultur dan ikan liar. Penyakit ini adalah umum di seluruh dunia dan menyebabkan kerugian berjuta dolar dalam industri ternakan ikan air tawar. Oleh itu, kajian dilakukan untuk mengenalpasti ciri-ciri morfologi, biokimia dan fisiologi *A. hydrophila* yang dipencilkan daripada ikan air tawar yang berpenyakit di negeri Selangor, Malaysia. Kesemua sampel ikan menunjukkan tanda-tanda klinikal seperti hemoraj pada kulit, mata cengkung dan sirip reput. Empat puluh isolat *A. hydrophila* yang dipencilkan daripada 150 ekor ikan liar dan ikan ternak menggunakan teknik coretan terus daripada kulit, ginjal, dan hati ke atas agar soya triptikase. Sampel ikan yang digunakan untuk kajian ini adalah tilapia hitam, *Oreochromis mossambicus* (n=30); tilapia merah hibrid, *Oreochromis* sp. (n=30); puyu kukum, *Scortum barcoo* (n=25); lampam jawa, *Puntius gonionotus* (n=10); jelawat, *Leptobarbus hoevenii* (n=10), patin, *Pangasius pangasius* (n=10); puyu, *Anabas testudineus* (n=10); keli Afrika, *Clarias gariepinus* (n=20) dan Louhan, *Cichlasoma* sp. (n=5). Kesemua isolat dikenalpasti menggunakan kit komersil, API 20E dengan ujian tambahan seperti oksidase, katalase, agen vibriostatik, toleransi NaCl, toleransi suhu dan aktiviti hemolitik. Secara morfologi, kesemua isolat adalah rod pendek, Gram negatif dan motil. Ujian biokimia menunjukkan hasil positif untuk ujian oksidase, katalase, ONPG, ADH, LDC, penggunaan sitrat, penghasilan indol, gelatinase dan Voges Proskauer. Selain itu, hasil negatif diperolehi daripada ODC, H_2S, urease dan TDA. Asid terhasil daripada glukosa, manitol dan arabinosa. Tiada penghasilan asid daripada inositol, sorbitol, ramnosa, sakarosa, melibiosa dan amigdalin. Semua isolat hidup dalam 0% dan 3% NaCl. Kemudian, bakteria isolat juga hidup pada suhu 22ºC dan 37ºC. Semua isolat menunjukkan β-hemolisis pada agar darah kuda. Isolat *A. hydrophila* dikenalpasti dengan lebih lanjut menggunakan penjujukan 16S rDNA.

Pada kajian ini, tindak balas rantaian polimerase (PCR) dilakukan untuk mengesan kehadiran gen virulensi pada 40 isolat *A. hydrophila* yang dipencilkan daripada ikan air tawar. Sepuluh gen virulensi tersebut adalah sitotonik labil haba (*alt*), sitotonik stabil haba enterotoksin (*ast*), sitotoksik labil haba enterotoksin (*act*), hemolisin (*hly*), aerolisin (*aer*), flagela A and flagela B (*fla*), lipase (*lip*), elastase (*ela*), serina protease (*ser*), and DNases (*exu*)) pada isolat. Kesemua isolat mempunyai gen *lip*, *exu* dan *ser*. Namun, tiada isolat yang mempunyai gen *ela*, *alt* dan *laf*. Hampir kesemua isolat mempunyai gen *hly* (95%) dan *aer* (75%). 30% kekerapan gen *ast* dan *act* dalam isolat *A. hydrophila.* Dua belas isolat mempunyai kombinasi gen tertinggi (*lip*+, *exu*+, *ast*+, *act*+, *hly*+, *aer*+, *ser*+) manakala dua isolat mempunyai kombinasi gen yang paling rendah (*lip*+, *exu*+, *ser*+).

Aeromonas hydrophila yang dipencilkan daripada ikan Louhan (AHFH40) yang mempunyai kombinasi gen virulensi yang tertinggi (lip^+, exu^+, ast^+, act^+, hly^+, aer^+, ser^+) dipilih untuk dijangkitkan pada *Oreochromis* sp. secara eksperimen. Juvenil *Oreochromis* sp. (bersaiz 4 inci) yang sihat disuntik secara intraperitoneal dengan 0.1 ml daripada larutan yang mengandungi 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 cfu/ml *A. hydrophila*. Tahap ketahanan pada jangkitan eksperimen dinyatakan sebagai LD_{50} menggunakan kaedah Reed dan Muench (1938). Parameter hematologi dan biokimia diperolehi daripada ikan kawalan dan ikan dijangkitkan secara eksperimen pada hari 1, 3, 5 dan 7. Penurunan yang signifikan diperhatikan pada haemoglobin tujuh hari selepas inokulasi *A. hydrophila*. Nilai min RBC, PCV dan Hb adalah berubah-ubah, tetapi rendah dengan signifikan (P<0.05) berbanding kawalan pada hari ke 3, 5 dan 7 selepas jangkitan. Ikan yang dijangkiti menunujukkan penurunan nilai min MCHC dan MCH pada hari ke 5 dan 7 selepas jangkitan jika dibandingkan dengan kumpulan kawalan. Nilai min WBC ikan yang dijangkiti mempunyai perubahan yang lebih tinggi secara signifikan pada hari ke 5 dan 7 selepas jangkitan. Min indeks biokimia aras TP, ALT dan AST juga berubah-ubah, dan berbeza secara signifikan (P<0.05) antara kumpulan yang dijangkitkan dan kawalan. Aras glukosa ikan yang djangkitkan meningkat secara signifikan (P<0.05) daripada nilai min kawalan. Ikan yang menunjukkan tanda-tanda klinikal MAS digunakan untuk penilaian histopatologi. Tisu daripada ginjal, hati dan limpa diawetkan di dalam 10% larutan formalin tampan, diproses dan diletakkan ke dalam blok lilin paraffin. Potongan lilin setebal 3-4 µm dilakukan dan diwarnakan dengan hematoksilin dan eosin. Hasil kajian ini menunjukkan LD_{50} *A. hydrophila* pada *Oreochromis* sp. dianggarkan antara 10^4 dan 10^5 cfu/ml. Perubahan histopatologi menunjukkan infiltrasi sel darah putih, nekrosis dan hemoraj pada ginjal, hati dan hepatopankreas yang mungkin disebabkan oleh sitotoksin yang dihasilkan oleh bakteria tersebut.

Kajian juga dilakukan untuk mengetahui tahap sensitiviti antibiotik, profil plasmid dan pengesanan integron *A. hydrophila* dilakukan. Isolat diuji dengan 10 jenis antibiotik iaitu penisilin G, sefaleksin, florfenikol, streptomisin, kanamisin, erythromisin, ampisilin, gentamisin, oksitetrasiklin dan tetrasiklin. Indeks kerintangan antibiotik pelbagai (MAR) dikira untuk menentukan tahap penggunaan antibiotik oleh penternak ikan. Tindak balas rantaian polymerase (PCR) dilakukan untuk mengesan gen integrase *Int1*, *Int2* dan *Int3*, gen tatasusunan kaset, gen terhubung-integron *aadA*, *sul1* and *qac1*, gen rintang streptomisin *strA-strB*, gen rintang β-lactamase bla_{TEM} and bla_{SHV}, dan gen rintang tetrasiklin *tetA-E* dan *tetM*. Dalam kajian ini, *A. hydrophila* adalah sensitif

terhadap eritromisin, florfenikol, kanamisin dan oksitetrasiklina, manakala ia adalah rintang terhadap sefaleksin, gentamisin and penisilin-G. Indeks kerintangan antibiotik pelbagai (MAR) pada isolat adalah dalam linkungan antara 0.4 to 0.5. MAR menunjukkan kawasan ternakan ikan tersebut mungkin mengalami pendedahan berterusan kepada antibiotik semasa peringkat pengkulturan ikan tersebut. Dua belas daripada 40 isolat mengandungi DNA plasmid bersaiz 6 hingga 23 kb. Terdapat hanya sedikit perbezaan pada saiz plasmid. Oleh itu, tiada profil plasmid khusus adalah dijangka untuk corak khusus kerintangan antibiotik. Gen *intl*1 dikesan daripada 50% isolat *A. hydrophila* (20/40) tetapi tiada isolat yang mempunyai gen *intl*2 dan *intl*3. Tiada kaset gen dikesan daripada semua isolat *A. hydrophila*. Gen *aadA* dijumpai pada 5/40 (12.5%) *A. hydrophila*, manakala kedua-dua gen rintang tetrasiklina; *tet*A dan *tet*C dijumpai pada 16/40 (40%) isolat *A. hydrophila.* Gen *strA*-*strB*, bla_{TEM} dan bla_{SHV} tidak dikesan pada mana-mana isolat. Oleh itu, kajian ini adalah laporan yang pertama untuk pengesanan integron pada isolat *A. hydrophila* di Malaysia.

Dapatan kajian ini menunjukkan jangkitan *A. hydrophila* merupakan isu kesihatan yang penting di ladang ternakan ikan. Peningkatan Aeromonads yang rintang antibiotik mengakibatkan cabaran untuk merawat jangkitan *Aeromonas*. Kajian ini juga menunjukkan kombinasi berbeza bagi gen virulen isolat *A. hydrophila* yang berperanan dalam patogenesis jangkitan *Aeromonas*. Hasil kajian hematologi dan histopatologi memberikan maklumat penting berkaitan patologi Aeromoniasis pada *Oreochromis* sp. Pemantauan berterusan perlu dilakukan untuk mengumpulkan lebih banyak maklumat *Aeromonas* spp. dan spesies bakteria akuatik lain bagi menghalang kejadian '*superbug*' antibiotik. Pengenalpastian penentu gen rintang pada *A. hydrophila* mempunyai kepentingan kesihatan awam dan alam sekitar.

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I certify that a Thesis Examination Committee has met on 24 April 2017 to conduct the final examination of Ruhil Hayati binti Hamdan on her thesis entitled "Characterization and Pathogenicity of *Aeromonas hydrophila* Isolated from Freshwater Fishes" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aadA	Streptomycin resistance gene
act	cytotoxic heat-labile enterotoxin
ADH	Arginine dihydrolase
aer	Aerolysin gene
ALT	Alanine transaminase
alt	Cytotonic heat-labile enterotoxin gene
AST	Aspartate aminotransferase
ast	Cytotonic heat-stable enterotoxin gene
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
bla_{TEM}, bla_{SHV}	β-lactamase resistance genes
cfu	Colony-forming unit
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
dpi	Days post inoculation
EDTA	Ethylenediaminetetraacetic acid
ela	Elastase gene
exu	DNases gene

FAO	Food and Agriculture Organization of the United Nations
fla	Flagella genes
H&E	Hematoxylin and eosin
hly	Hemolysin gene
HCl	Hydrochloric acid
H_2S	Hydrogen sulphide
int	integron gene
LD_{50}	Median lethal dose
LDC	Lysine decarboxylase
lip	Lipase gene
MCHC	Mean corpuscular haemoglobin concentration
MCH	Mean corpuscular hemoglobin
NaOH	Sodium hydroxide
NaCl	Sodium chloride
ODC	Otnithine decarboxylase
ONPG	O-Nitro Phenyl Galactosidase
PCR	Polymerase chain reaction
PCV	Packed cell volume
*qac*1	Quarternary ammonium compound resistance gene
RNA	Ribonucleic acid

rRNA	Ribosomal ribonucleic acid
RSA	Rimler – Schotts agar with ampicilin
HBA	Horse blood agar
RBC	Red blood cell
*str*A-*str*B	Streptomycin resistance genes
*sul*1	Sulphonamide resistance gene
TBE	Tris-borate-EDTA
TDA	Tryptophane deaminase
*tet*A-E, *tet*M	Tetracycline resistance genes
TP	serum total protein
TSA	Trypticase soy agar
TSB	Trypticase soy broth
UV	Ultra violet
w/v	Weight per volume
w/w	Weight per Weight

CHAPTER 1

INTRODUCTION

In 2012, Ministry of Agriculture declared aquaculture as potential food industry of Malaysia. This sector has been included into the country transformation programme under the National Key Economic Area, NKEA (Department of Fisheries, 2014). The percentage of aquaculture production has seen an increase year by year. The total freshwater aquaculture production showed an increment of 19.7 per cent from 163,8000 tonnes in 2012 to 196,1000 metric tonnes in 2013 (Department of Fisheries, 2014). Tilapia is an important freshwater fish among the global aquaculture. Annual tilapia production has been increasing two- or threefold every decade since 1990 (Bhujel, 2014). In Malaysia, it became the second highest aquaculture production compared to other freshwater fishes (Department of Fisheries, 2015). It is the most profitable fish species and tilapia industry rapidly expanded over the years due to the increase in demand. During Chinese New Year, a high demand for live tilapia in retail market and seafood restaurants in Malaysia, Singapore and Taiwan was observed. In Malaysia, price of live tilapia is usually at around US$7/kg. Nevertheless, during the festive season some restaurants tagged the price at US$15/kg. Supplies in Malaysia come almost entirely from local sources, in addition to live tilapia exports to Singapore. In fact, approximately 1,500 tonnes of tilapia from Malaysia is exported to Singapore annually (Infofish International, 2016).

The progressive production has led to inapt farm management systems viz. high stocking density, fluctuating temperature, poor aeration and water exchange that led to stress and increase in the susceptibility to microbial infections. Diseases are major hinderances in the development and sustainability of aquaculture practices throughout the world. Bacterial pathogens are among different types of infectious agents that often responsible for severe mortalities in a wide range of fishes at different stages of growth (Swain *et al*., 2002). As a result, farmed Tilapia is facing several disease outbreaks e.g., motile aeromonas septicaemia, streptococcosis and edwardsiellosis (Lukkana *et al*., 2012). The consequence of fish diseases resulted in financial loses and unmarketable appearance of infected fish (Sreedharan *et al*., 2012).

Motile aeromonas septicaemia (MAS) is considered a major constraint to aquaculture production globally. Motile aeromonas septicaemia can be acute, chronic or latent with signs that include small pinpoint hemorrhages at the base of fins or on the skin, surface lesions, exophthalmia, abdominal distension, muscle necroses and dermal ulcers. Infected fish also frequently exhibit internal signs including fluid in the abdomen, swollen liver and spleen; and distended fluid filled intestines (Pachanawan *et al*., 2008). Fish pathogenic motile aeromonads have been usually linked with *Aeromonas hydrophila* (Austin and Austin 2012; El – Sayed, 2006). The prevalence of *A. hydrophila* infections in cultured Tilapia seems to be higher than the wild fish and the mortality rate could be very high up to 80–100% (Faisal *et al*., 1989). Depending on host species, strain or dose of bacteria and environmental conditions (e.g. temperature,

poor water quality, overcrowding, and rough handling) make fish more susceptible to the disease.

The order Aeromonadales comprises of a single family of bacteria, Aeromonadaceae, where the genus Aeromonas resides (Martin and Joseph, 2005). *Aeromonas hydrophila* is designated as the type species. *A. hydrophila* is a Gram-negative and naturally occurring inhabitant of aquatic environments, namely freshwater, marine water and estuarine water. This bacterium is an opportunistic pathogen in fish, reptiles and humans. However, in some situations it may be a primary pathogen with evidence suggesting adverse environmental conditions or other compromising factors such as the presence of high levels of parasites can be conducive to establish infection and disease in fish (Liu and Lu, 2004). *A. hydrophila* is a zoonotic agent which can be transmitted from animals to human and vice versa (Pachanawan *et al*., 2008). The bacteria also can be conveyed to humans by ingestion of contaminated water and food viz seafood, raw meat, vegetables and milk. *A. hydrophila* is considered a foodborne pathogen of emerging importance attributable to its ability to grow at refrigerated temperature (Daskalov, 2006).

β-haemolysin, aerolysin, extracellular lipase, cytolytic enterotoxin, haemolytic toxin and extracellular protease are several extracellular products (ECP) of *A. hydrophila* and have been suggested as possible contributory factors in its pathogenesis (Chu and Lu, 2005). The studies on virulence characters provide useful information with regard to the pathogenicity of *A. hydrophila.* The polymerase chain reaction (PCR) has provided a rapid and highly sensitive method for the detection of pathogenic microorganisms from diseased fish (Raja *et al*., 2004). In addition, PCR detection of virulence genes has been used to identify potentially pathogenic *Aeromonas* isolates (Hu *et al*., 2012). Puthucheary *et al*. (2012) stated that virulency in *Aeromonas hydrophila* is multifactorial, which consists of cytotonic heat-labile, cytotonic heat-stable enterotoxins, cytotoxic heat-labile enterotoxin, hemolysin, aerolysin, flagellin, lipase, elastase, serine protease, and DNases play significant role in pathogenesis. Thus, many studies on the virulence factors of *A. hydrophila* have been performed using strains isolated from fish (Chu and Lu, 2005; Nam and Joh, 2007; El – Barbary, 2010; Li *et al*., 2011; Hu *et al*., 2012; Onuk *et al*., 2013; Hussain *et al*., 2014).

Fish health management can be achieved through good husbandry, rapid detection of pathogens and control by antibiotics, vaccination, immunostimulants and phytotherapy. Rapid disease diagnosis plays a crucial role in fish health management. Detection of fish pathogens can be done by using conventional (laboratory culture and identification), histology, immunological and molecular methods. A combination of methods is often required for a definitive diagnosis of a disease (Adams, 2004).

Antibiotics are substances that kill or inhibit the growth of bacteria. The undiscerning use of these compounds has induced antimicrobial resistance. Thus, bacterial infection will become even more difficult to treat in the future (Sarria-Guzmản *et al*., 2014). Moreover, the accumulation of antibiotics both in the environment and in fish can be hazardous to consumers and the environment. Nowadays, an increase in antibiotic

resistance of *Aeromonas* spp. strains due to the irrational use of drugs to control infections in fish farms has been reported (Nguyen *et al*., 2014). This generates selection pressure on microorganisms and consequently induces the acquisition of resistance toward antibiotics. Thus, the search for genetic elements associated to antibiotic resistance in microorganisms becomes more important. Plasmids, transposons, and integrons can carry antibiotic – resistant genes and can be transferred horizontally between microorganisms. Integrons have become very important recently as they can capture more than one antibiotic-resistant cassette (Lukkana *et al*., 2012).

1.1 Statement of Problems and Significance of Study

Aeromonas hydrophila infection is the scourge of fresh and warm water fish farming worldwide and is considered as a significant economic problem, particularly in China and India over the past decade. It is also believed to be a pathogen of emerging importance for humans through consuming fish and shellfish contaminated with *A. hydrophila*. However, the principal concern is in the intensive culture of fish, since the bacteria can inflict severe losses and can present a risk of infection not only for the fish but also for handlers and consumers.

In 2011, an outbreak was reported in fish farm at Bagan Lalang, Sepang, Selangor. Jade perch, *Scortum barcoo* is the species reared in the farm using recirculating aquaculture system (RAS). The farmer reported the case to Aquatic Animal Health Unit (AAHU) in UPM. The fish showed clinical signs of skin hemorrhage, sunken eyes and fin rot. The symptoms are believed to be caused by bacteria. Therefore, more attention should be paid to improving diagnostics techniques by using molecular techniques such as polymerase chain reaction (PCR). The pathogenicity of *A. hydrophila* may involve several extracellular products including proteases, hemolysins and enterotoxins. Further research is required to determine the virulent factors responsible for infection in fish.

An effective control of this bacterium through administration of appropriate antibiotic is also important to combat the diseases. Currently, the irresponsible manner usage of antibiotics may complicate health management by triggering toxicity, resistance, residues and occasionally public health and environmental consequences. Bacteria can even transfer their drug resistance to other bacteria and causing more problems. In Malaysia, lack of study to detect integron and antibiotic resistant genes in *A. hydrophila* from diseased freshwater fishes.

1.2 Hypotheses of Study

Aeromonas hydrophila is a pathogenic aquatic bacteria for Red hybrid tilapia. It can be detected via morphological or standard methods (laboratory culture and biochemical identification) and molecular methods. *Aeromonas hydrophila* have different combination of virulence genes. The severity of lesion in the experimental fishes attributed to toxic substance produce by *A.hydrophila* which evoked hemorrhage and necrosis. Infection-causing bacteria can become resistant to multiple antibiotics. Integron can be detected in *A. hydrophila* isolated from freshwater fishes.

1.3 Objectives of the study

The objectives of the present study were as follows:

1. To characterize *A. hydrophila* isolated from freshwater fishes morphologically, biochemically and physiologically.
2. To detect virulence genes in the *A. hydrophila* isolated from freshwater fishes.
3. To determine the pathogenicity of *A. hydrophila* in red hybrid tilapia, *Oreochromis* sp.
4. To determine antibiotic sensitivity, plasmid profiling, integron and antibiotic resistance genes of *A. hydrophila* isolates.

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