PENERBIT PRESS
UNIVERSITI TEKNOLOGI MARA
eISSN: 2785-924X
2022 March, Volume 2, Issue 1

Journal of Smart Science and Technology

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Journal of Smart Science and Technology
PENERBIT PRESS UNIVERSITI TEKNOLOGI MARA
2022 March Volume 2 Issue 1
eISSN: 2785-924X

Pathogenicity of *Aeromonas hydrophila* in Cultured African Catfish (*Clarias gariepinus*)

Nora Faten Afifah Mohamad[1], Hassan Mohd Daud[1], Sharifah Raina Manaf[2,*]

[1]Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Cawangan Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia

Received: 18-01-2022
Revised: 07-03-2022
Accepted: 23-03-2022
Published: 30-03-2022

*Correspondence
Email: sharifahraina@uitm.edu.my
(Sharifah Raina Manaf)

DOI: https://doi.org/10.24191/jsst.v2i1.25

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Abstract
Aeromonas infections are becoming a serious risk issue in commercial aquaculture, and a wide range of fish and shellfish species has been documented as being vulnerable. Five isolates of Aeromonas hydrophila were identified from African catfish (Clarias gariepinus) cultured in Selangor, West Malaysia in this study. A conventional rapid identification approach (API 20E strip) was used for preliminary identification based on the biochemical properties of the isolated bacteria. Polymerase chain reaction (PCR) with the specific primer 16 rDNA, on the other hand, was used as an accurate and confirmed identification. A pathogenicity test via intramuscular (IM) injection was used to investigate the virulence of A. hydrophila. With a high degree of similarity (98%) to the NCBI or Genbank databases, the isolates were identified as A. hydrophila. The LD_{50} was calculated using pathogenicity test findings and was found to be $2.1 \times 10^{6.33}$ CFU mL^{-1}, while 1×10^{8} CFU mL^{-1} in the experimentally injected fish, resulted in 100% mortality. Several organs, including the kidney, liver, and spleen, showed histopathological abnormalities. Those changes mainly include increase in the presence of hemosiderin deposits, congested portal vessels, vacuolated hepatocytes, generalised loss of tubular cells, and oedematous degeneration in the infected organs.

Keywords
Aeromonas hydrophila; African catfish; Pathogenicity; Polymerase chain reaction; Malaysia

Citation: Mohamad, N. F. A., & Daud, H. M., & Manaf, S. R. (2022). Pathogenicity of *Aeromonas hydrophila* in cultured African catfish (*Clarias gariepinus*). *Journal of Smart Science and Technology*, *2*(1), 34-45.

1 Introduction

Nowadays, the technologies of aquaculture systems in Malaysia are well-developed. Many farms have already implemented intensive culture systems both in the hatchery and grow-out phases. However, poor management practices in fish farming such as deprived disposal of fish waste, inadequate workers and lack of technical knowledge among farmers especially in intensive culture systems, may trigger the emergence of diseases and often lead to massive death of cultured fish in fish farms[1]. In freshwater cultured fish as well as marine fish culture, disease outbreaks due to various pathogenic bacteria are very common resulting in

substantial annual economic losses to farmers in the aquaculture industry. Among the common Gram-negative bacteria pathogens that adversely impact fish in culture systems are *Aeromonas*, *Edwardsiella*, *Flavobacterium*, *Francisella*, *Photobacterium*, *Piscirickettsia*, *Pseudomonas, Tenacibaculum*, *Vibrio* and *Yersinia*[2-4].

Among these microbial pathogens, *Aeromonas hydrophila* is known as one of the most harmful bacterial which is the causative agent of motile *Aeromonas septicemia* (MAS) in fish[5,6]. The disease outbreaks were associated with heavy mortalities in wild and farmed fishes globally[7]. In Malaysia, *A. hydrophila* is commonly reported as a major cause of mortality in the industry[8]. The first case of mass mortality reported in an African catfish farm identified the *A. hydrophila* as the causal agent[9]. The authors suggested that the disease outbreak was triggered by the role of β−hemolysis and aerolysin as the virulence factor in the bacteria, in addition to other environmental stress.

The objectives of this research were to isolate phenotypic as well as genotypic characterization of *A. hydrophila* from moribund African catfish (*Clarias gariepinus*) with clinical features and prognosis of *Aeromonas* septicaemia, and to test its pathogenicity on experimentally infected catfish via intraperitoneal injection.

2 Method

2.1 Sampling of Catfish and Bacterial Isolation

A total of 45 moribund African catfish, *C. gariepinus* were collected from five different private fish farms located in Selangor, Malaysia as shown in Table 1. Management practices are almost similar among fish farmers in some instances, which were associated with occurrence of deaths in fish. All sampled fish showed clinical signs of skin ulcers, hemorrhages and fin rots. Samples of the kidney, liver, gills, spleen tissues and lesions from skin were used for the isolation of the *Aeromonas* sp. and Rimler-Shotts Agar (RSA) (HIMEDIA, India) was utilised and incubated at 28 ± 2°C for 18 to 24 hours. The culture medium was prepared according to the instructions of the manufacturer by dissolving 45.43 g to 990 mL of distilled water. The plates were then examined for bacterial growth. Dominant colonies were selected and reinoculated on sterile TSA (Oxoid, UK) medium until single colonies were obtained. Further identification was followed by gram-staining, catalase and oxidase tests to confirm the identification. Strains showing catalase-positive and Gram-negative bacilli were subcultured in TSA and incubated overnight to give pure colonies.

API 20E strips (Biomerieux, France) were used to determine the biochemical properties of a bacterial isolate from *C. gariepinus*, according to the manufacturer's instructions. A well-known isolate of *A. hydrophila* was used to quality control and validate the traditional API 20E (ATCC 35654 strain). The bacterial genus and species were classified using the approach explained in the Bergey's Manual of Systematic Bacteriology[10]. In addition, a single colony of isolate was streaked across blood agar (Sheep rbc) plates using sterile inoculating wire loop for haemolytic activity. Plates were incubated at 37°C for 18 to 24 hours. After incubation, the haemolytic activities were determined by using haemolysis on blood agar.

2.2 Genotype Characteristics of Bacterial Isolates Using PCR Assay

The bacteria DNA was extracted using the 'PrestoTM Mini gDNA Bacteria Kit' from five isolates that were phenotypically similar to *A. hydrophila* isolates. A thermocycler was used to do DNA amplification on 5.0 µL of DNA extract in 25 µL (BioRad, UK). The primer was targeted at a species-specific region of the *A. hydrophila* 16S rRNA and the primer pair was shown in Table 2. PCR reaction was carried out by using 12.5 µL GoTaq® Green Master Mix (Promega, USA), 2.0 µL of the primer (16S rRNA) (1.0 µL forward and 1.0 µL reverse), 5.5 µL Nucleus Free Water (NFW), and 5 µL template DNA in an assay volume of 25 µL.

Table 1. List of the newly collected bacterial isolates from *C. gariepinus*.

Recovery site	Isolates Code	Source
Kidney	CG1K, CG2K, CG3K, CG4K, CG5K, CG6K, CG8K, CG9K, CG10K, CG12K, CG13K, CG14K, CG15K, CG16K, CG17K, CG18K, CG19K, CG20K, CG21K, CG22K, CG23K, CG24K, CG28K, CG31K, CG32K, CG33K, CG35K, CG36K, CG37K, CG38K, CG39K, CG40K, CG41K	i) Kim Seng Fish Farm, Seri Kembangan, Selangor ii) Victor's Fish Paradise, Semenyih, Selangor iii) Fish Farm, Batang Berjuntai, Selangor iv) Tasik Idaman, Bangi, Selangor v) Three Ocean Fish Pond & Trading, Rawang, Selangor
Liver	CG7L, CG25L, CG29L, CG44L, CG45L	i) Three Ocean Fish Pond & Trading, Rawang, Selangor ii) Victor's Fish Paradise, Semenyih, Selangor
Spleen	CG26S, CG27S, CG30S	i) Kim Seng Fish Farm, Seri Kembangan, Selangor
Skin	CG11SP, CG34SP, CG42SP, CG43SP	i) Kim Seng Fish Farm, Seri Kembangan, Selangor ii) Victor's Fish Paradise, Semenyih, Selangor

The PCR program for DNA amplification were as follows: initial denaturation at 95°C for five minutes followed by 30 cycles at 95°C for two minutes, primer annealing at 55°C for one minute, DNA extension at 72°C for one minute and final extension at 72°C for seven minutes. Afterwards, the PCR products were electrophoresed on agarose 1.5% agar at 100 V for 45 minutes, after which the gel was lifted, visualized and captured by using Bioimaging System, Gel Doc™ EZ Imager (BioRad, US). QIA quick PCR purification kit was used to purify the PCR products (QIAGEN-USA), which were then sent to 1st BASE CO (DNA sequencing service) Malaysia to sequence purified PCR products directly.

2.3 *Lethal Median Pathogenicity Test, ($LD_{50\ at\ 96h}$) and Histopathological Examination*

Healthy African catfish, *Clarias gariepinus* (TL: 17.36 ± 1.72 cm, BW: 13.05 ± 3.16 g) were collected from the hatchery of Aquatic Animal Health Unit (AAHU), Faculty of Veterinary Medicine, Universiti Putra Malaysia. For two weeks, the fish were acclimatized to laboratory conditions in a 1,000 litres fibre-tank with water temperature of approximately 24 ± 1°C. They were fed with *ad-libitum* twice, daily with a commercial fish diet based on 5% of the body weight. The fish diet formula consisted of crude protein (32%), lipid (3%), and moisture content (12%) which were manufactured by Star Feedmills (M) Sdn. Bhd. Prior to experiments, the fish were fasted for 24 hours and starved during the experiment period.

Table 2. The 16S rDNA of *Aeromonas hydrophila* was detected using oligonucleotide primers.

Primer	Identification (5'-3')	Size in base pair (bp)	Reference
16 rDNAf	GAAAGGTTGATGCCTAATACGTA	685	Nielsen et al.[12]
16 rDNAr	CGTGCTGGCAACAAAGGACAG		

In the pathogenicity test, a virulent strain of *A. hydrophila* obtained from catfishes with a Beta hemolytic characteristic was used. The pathogenicity test bacteria suspension was adjusted to McFarland turbidity (Standard no.5), which was equal to 2.1×10^9 cfu mL^{-1}. Intraperitoneal injection (IP) of 0.1 mL of five serial dilutions (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5}) of *A. hydrophila* into juveniles of *C. gariepinus* was determined by the 96 h-LD_{50}. The experiment was carried out for 96 hours with ten fish per treatment (in triplicates). Prior to injection of 0.1 mL *A. hydrophila*, all fish were fasted overnight and anesthetized in a 50 to 70 mL^{-1} Tricaine Methanesulfonate (MS−222) (Sigma-Aldrich, St. Louis, US) for one minute. Then, IP injections were carried out according to different concentrations while normal saline was used as control treatments. Water quality including temperature, pH and DO levels of each aquarium was measured daily using a multi−parametric probe (Hanna, model HI 8424) twice, daily in the morning (8.00 am) and evening (4.00 pm) to check for any changes that would affect the results. The values recorded for temperature, pH and DO level were 26 to 27˚C, 7.0 to 8.0 and 5.5 to 6.0 mgL^{-1}, respectively. One−third of the water volume in the tanks and aquaria was also changed on a daily basis to avoid water deterioration.

The experiment was carried out for a period of 96 hours post-challenged, and mortality of the inoculated fish were monitored at various time intervals and recorded promptly. Dead fish were also collected daily and quickly removed from the tanks. Susceptibility to experimental infection was expressed as $LD_{50\text{-}96h}$ calculated by the method as stated below[11]:

$$\log LD50 = \alpha \log b + c \quad (1)$$

where α = mortality>50%-50%, mortality>50%-mortality<50%, b = dilution ratio (10^{-1}) and c = logarithms of minimum dilution ratio in which the mortality was > 50%.

Infected fish showing clinical signs of Motile *Aeromonas* Septicemia (MAS) and one fish in the control group were sacrificed and immediately dissected after being experimentally infected with *A. hydrophila*. The samples of liver, kidney and spleen were taken and fixed in 10% buffered formalin for at least more than 24 hours and further processed for histopathological examination. Then, the fixed tissues were processed in an automatic tissue processor (Leica TP 1020, Germany) involving the process of dehydration, clearing and wax infiltration. The tissues were then embedded in paraffin wax, and were sectioned using a rotary microtome (4−5 µm) (Leica RM−2245, Germany). The ribbon with the thin sections was placed on a water bath at a temperature of 40˚C, which were finally picked up over glass slides. After staining with standard haematoxylin and eosin (H&E), the sections were mounted on clean glass slides. After allowing the stained slides to dry overnight, they were viewed under a light microscope (Nikon, Eclipse, E 800) to study for any pathological changes in tissues after bacterial infection.

3 Results

3.1 Bacterial Isolation

On TSA plates, the isolated colonies were yellowish opaque, spherical, convex with smooth edges, and semi-translucent (Figure 1(a) and Figure 1(b)). Gram negative, short rod shaped, motile, oxidase positive, catalase positive, and fermentative isolates were isolated from *C. gariepinus*, implying that colonies could be *Aeromonas*.

3.2 Phenotypic Characterization of A. hydrophila

A. hydrophila colonies on RSA agar were black, round and convex (Figure 1(c)) while all isolates showed β-haemolysis on

horse blood agar (Figure 1(d)) which indicates the presence of hemolysin enzyme to degrade the blood. By using standard biochemical characteristics and the API 20E technique, the bacterial isolates were also identified to the species level as *A. hydrophila*. All positive and negative results of the biochemical characteristics of the isolates performed by using conventional method and API 20E are summarized in Table 3. The rapid API 20E system effectively recognised *A. hydrophila* isolates by generating a profile number that corresponded to the *A. hydrophila* profile number in the API 20E handbook.

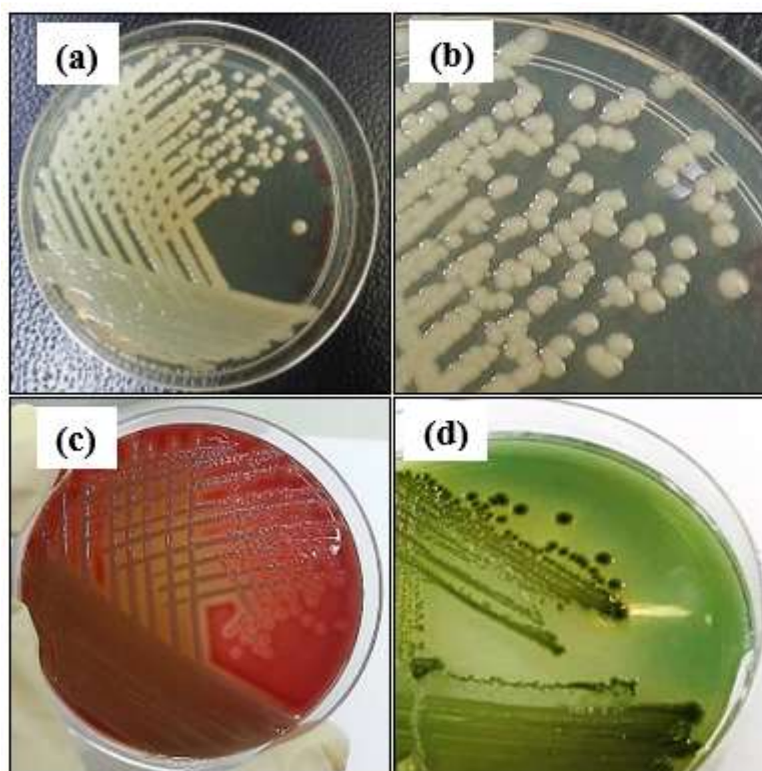

Figure 1. Morphological characteristics of *A. hydrophila*. (a−b) Single colonies of *A. hydrophila* on TSA. (c) *A. hydrophila* on horse blood agar (d) *A. hydrophila* colonies on RSA.

3.3 Aeromonas Hydrophila Genotypic Characterisation by PCR Assay and Sequencing Findings

Amplification of 16S rRNA gene in all bacteria isolates produced a 685 bp amplicon as expected (Figure 2). The BLASTn result of 16S rRNA gene showed all *A. hydrophila* strain species with 98 to 100% similarities to NCBI GeneBank nucleotide sequences database. The PCR primers designed by Nielsen et al.[12] for specific detection of *A. hydrophila* were tested successfully and a desired PCR product of 685 bp was obtained in reaction containing genomic DNA *A. hydrohila.*

3.4 Pathogenicity and Histopathological Alteration Induced by A. hydrophila

The majority of African catfish, *C. gariepinus*, that were given a high dose of bacteria (10^7-10^9) died within three days. Experimentally infected catfish showed disease symptoms that were identical to infected catfish reported in commercial private farms. Figure 3 and Figure 4 depicts the clinical symptoms that were seen. Some catfish, on the other hand, died without presenting any clinical indications. The infectivity of this isolate on the injected fish was confirmed by re−isolation of *A. hydrophila* from catfish with no clinical symptoms indicating that the pathogens are still transmissible despite the absence of visible clinical signs. The challenge test

was an experiment and the result revealed that the value of median lethal dose (96 h−LD_{50}) in *C. gariepinus* was $10^{6.33}$ and 100% mortality occurred in *C. gariepinus* injected with 2.1×10^8 cfu mL^{-1}. However, there was no mortality in the control group. On the other hand, based on the results of LD_{50}, the severity of motile septicemia caused by virulent *A. hydrophila* is also influenced by the level or the abundance of bacterial communities of *Aeromonas* in *Clarias gariepinus*.

Table 3. Morphological, physiological and biochemical characteristics of *Aeromonas hydrophila.*

Characteristic	Response of *A. hydrophila* isolates
Gram reaction	Negative
Shape	Rod
Motility	Positive
Physiological Test	
Growth at different temperature	
1. 4°C	−
2. 22°C	+
3. 37°C	+
4. 45°C	−
Growth in NaCl	
i. 0%	+
ii. 3%	+
iii. 7%	−
Biochemical Tests	
ONPG	+
Lysine decarboxylase	+
Arginine dihydrolase	+
Ornithine decarboxylase	−
Citrate	+
Tryptophane deaminase	−
Hydrogen sulfide	−
Urease	−
Indole	+
Voges – Proskauer	+
Gelatine	+
Glucose	+
Mannitol	+
Inositol	−
Sorbitol	−
Raffinose	−
Rhamnose	−
Melibiose	−
Sucrose	−
Saccharose	−
Arabinose	+
Oxidase	+
Catalase	+
O/129 sensitivity	−

Note: +: positive result; -: negative result; ONPG: O-Nitrophenyl-β-galactoside

C. gariepinus had histopathological abnormalities in multiple organs, including the kidneys, livers, and spleens (Figure 5). The kidneys were the most affected organ with multifocal hemosiderin presented in *C. gariepinus*. Irregular vacuolation of the cytoplasm of the hepatopancreas associated with the lipid degeneration was observed in *C. gariepinus.*

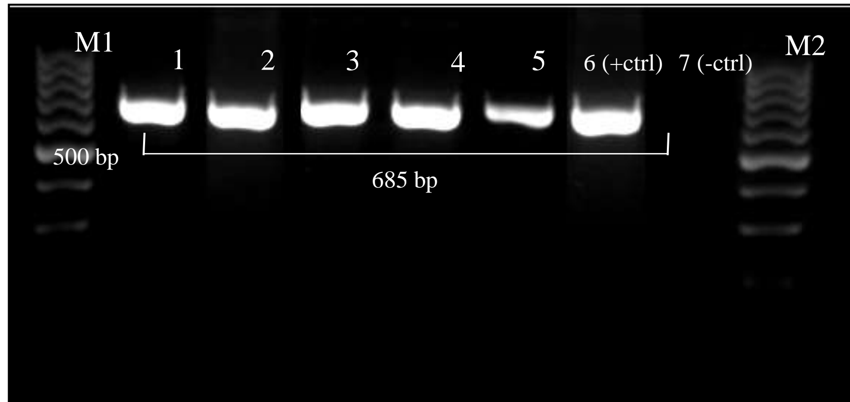

Figure 2. PCR amplicon (16S rDNA) amplified from bacteria samples. M1, M2: 100bp marker; Lane 1: *Aeromonas hydrophila* isolated from *Clarias gariepinus* from CG14K; Lane 2: *A. hydrophila* isolated from *Clarias gariepinus* from CG31K; Lane 3: *A. hydrophila* isolated from CG25L; Lane 4: A. hydrophila isolated from *C. gariepinus* from CG26S; Lane 5: *A. hydrophila* isolated from *C. gariepinus* from CG43SP; Lane 6: +ctrl: the stock culture of *A. hydrophila*; Lane 7: −ctrl). K=Kidney (CG14K), L=Liver (CG25L), S=Skin (CG26S), SP=Spleen (CG43SP).

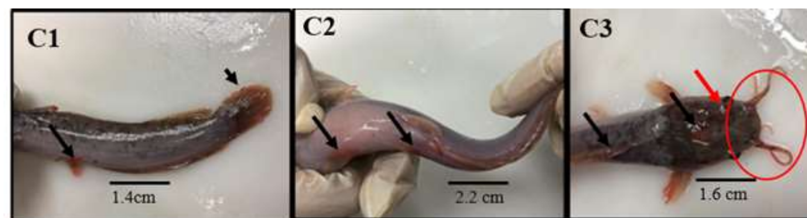

Figure 3. Clinical signs observed in moribund of *Clarias gariepinus* challenged with *Aeromonas hydrophila*. Haemorrhages at pectoral and pelvic fins base (black arrow) (A1, A2, A3); hyperaemia at the caudal fin (arrowhead) (A1); haemorrhages around the opperculum and barbels (red circle) (A3); exophthalmia (red arrow) (A3).

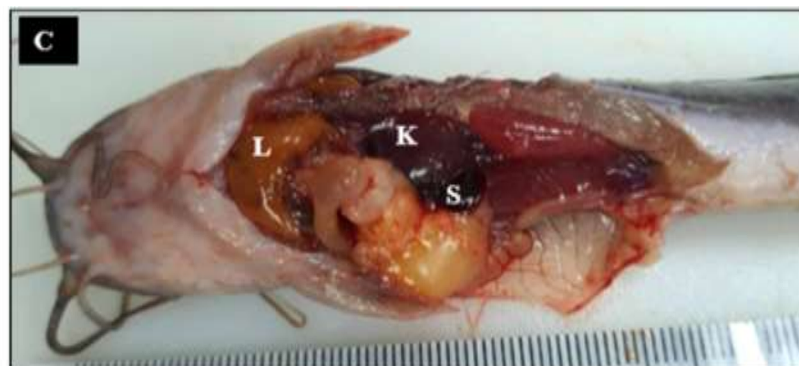

Figure 4. Internal organs of *Clarias gariepinus* challenged with 2.1 x 10^6 cfu mL^{-1} of *Aeromonas hydrophila* showing kidney (K) congestion and enlargement of spleen (S) with pale liver (L).

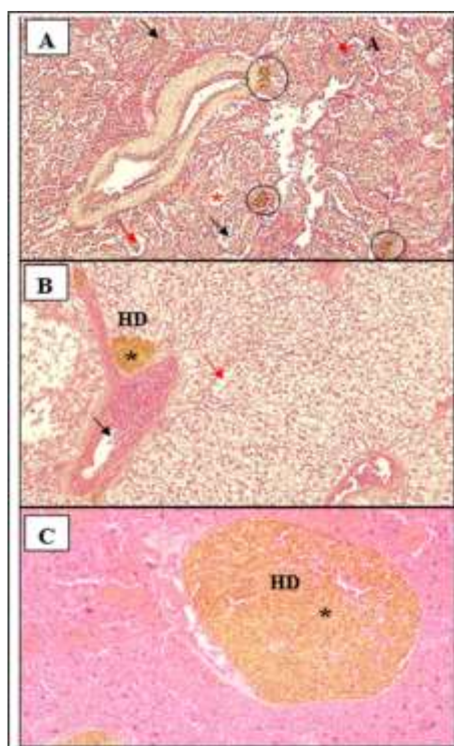

Figure 5. Histopathological changes in the kidney (A), liver (B) and spleen (C) of challenged *Clarias gariepinus* at 96 h post challenged. Infected kidney showed generalised loss of tubular cells, and glomeruli (A) (red arrow), oedematous degeneration (black arrow), increase presence of hemosiderin deposits (HD) (black circle). In liver, congested portal vessel (black arrow), vacuolated hepatocytes (red arrow); and presence of hemosiderin (*). In spleen, presence of a huge hemosiderin deposit (HD) (*). H & E. Mag. 400x.

4 Discussion

Aeromonas hydrophila is found in freshwater environments all over the world and considered to be opportunistic pathogens which frequently causes disease in many fish and aquatic organisms. In this present study, 45 isolates of *A. hydrophila* were obtained from diseased *C. gariepinus* from several private catfish farms. Freshwater environments, especially with an organic-rich freshwater, are usually thought to be natural habitats for *A. hydrophila*, but recent research from Japan and the United States suggests that it could also be a member of fish gut flora, mucosal surfaces and internal organs of clinical healthy fish[7,11]. Earlier report of *A. hydrophila* isolation in Malaysia were made by several researchers who isolated the bacteria from various freshwater fish species including *Anabas testudineus, Scortum barcoo*, *Oreochromis mossambicus, Puntius gonionotus, Leptobarbus hoevenii, Pangasius pangasius, Cichloma* sp. *Clarias gariepinus* and ornamental fish species from aquarium shops such as Dwarf gourami (*Colisa lalia*), Black tetra (*Gymnocorymbus ternetzi*), Silver catfish (*Pangasius sutchi*), Guppy (*Poecilia reticulata*), Discus cichlids (*Symphysodon* spp.), Tiger barb (*Barbus pentazona hexazona*) and Platy *(Xiphophorus maculates*)[13-15].

In agreement with the previous studies done by Janda et al.[16] and Jayavignesh et al.[17], biochemical characters found in the present study indicated that all isolates belonged to *A. hydrophila.* The biochemical profile of *A. hydrophila* in this study was also comparable to previous studies done by Whitman and MacNair[10] and Coz-Rakovac and Strunjak-Perovic[18]. Basically, many extracellular proteins which are also called virulence genes, were produced by

A. hydrophila including hidrolipase (*Lip*), elastase (*ahyB*), lipase (*pla/lip*), cytotonic enterotoxin (*alt*), cytotoxin enterotoxin (*act*), temperature sensitive preotease (*eprCA1*), serine protease (*Ahp*), cytotonic heat stable enterotoxin (*ast*), haemolysin (*hlyA*) and aerolysin (*aerA*)[19]. All of these virulence genes are associated with pathogenicity and environmental adaptability of the microorganism.

The main clinical signs of infected fish in the present study were associated with abnormal behaviours which were aggregated on the water surface and erratic swimming. These observations might be due to bacterial toxins as reported by Fang et al.[20]. The authors mentioned that the lethargic movement of infected blue gourami, *Trichogaster trichopterus* (Pailas) was possibly the result of erosion of tail, oedema, haemorrhages, and ulcerated fins which influenced the normal behaviour of the diseased fish. The symptoms observed in this study were similarly very comparable to those observed in other fish species such as channel catfish (*Ictalurus punctatus*), Red hybrid tilapia (*Oreochromis* sp.) and Golden mahseer (*Tor putitora*)[21-23].

In addition, dermal ulceration with focal hemorrhages and inflammation observed in current work indicated chronic infection by *A. hydrophila* and could be attributed to septicaemic reaction expressed by motile *aeromonas* infection[25]. Hyperaemic spots at the base and tips of fins in some of the infected fish seen in this study have also been reported in Indian catfish, *Clarias batrachus*[25] and Nile tilapia, *O. niloticus* which were experimentally infected with similar *Aeromonas* bacteria[26]. It could be due to depigmentation of the skin melanophores and/or melanophores which were related to anaemia[27-28]. Thus, external clinical presentation in all groups of infected fish from experimental infection suggested that *Aeromonas* strains used in this study established infection in all fish species, however, the severity varied and closely related to the species and immune status of fish challenged.

On the other hand, the post-mortem examination on the infected fish showed enlargement in the internal organs primarily in the liver, spleen and kidney which were similar to those reported in Red hybrid tilapia, *Oreochromis* sp.[29]. The authors described that the pathogenic effect of *A. hydrophila* infection and the most apparent clinical signs included petechiation of the liver and enlargement of spleen, accompanied by bilateral exophthalmos. The clinical signs observed on infected fish can be due to the bacterial invasion and colonization, and production of toxins produced by *A. hydrophila*[30-31].

Histopathological alterations observed in the present study showed hepatic tissues with abnormal hepatocyte morphology represented by vacuolation, an atrophy which is undergoing necrosis. The results were comparable with earlier investigations related to infection of *A. hydrophila* on other freshwater fish species[32-36]. These pathological alterations were associated with toxins and extracellular products such as haemolysin, protease and elastase produced by *A. hydrophila*[9,31]. Other than that, morphological modifications, such as the vacuolization of the hepatocytes found in this study can be a signal of degenerative process associated with metabolic damage and hepatic disturbances in the fat metabolism[37,38].

Kidney tissues also showed degeneration of tubular cells and deposition of hemosiderin as gold-brown pigments. Some researchers reported similar pathological changes in the kidney and postulated that it was related to interstitial nephritis which affected the kidney's function and led to losses in its structural integrity[39-41]. Not only that, the presence of numerous hemosiderin in the kidney and spleen produces a condition known as hemosideriosis. In addition, hemosiderosis was also associated with chronic inflammatory lesions[42]. These characteristic features of hemosiderosis were also found in crucian carp which indicated that β-haemolysin secreted by *A. hydrophila* caused haemolysis inside the fish body followed by deposition of haemosiderin[43]. Wolke et al.[44] first suggested that occurance of MMCs was associated with stressful condition of the fish and played an important role as an indicator to monitor fish health. Hemosiderin is a distinct grouping of four

forms of brown pigments, including melanin, lipofuscin, ceroid, and hemosiderin, that has multiplied into MMCs[45] and normally located in the stroma of the hematopoietic tissue of the kidney[42,46] as in the case of the present study.

5 Conclusion

In summary, the present study clearly showed that *A. hydrophila* was found to be highly infectious that could cause severe septicemic effect in African catfish. The mortality caused by *A. hydrophila* also depends on the concentration of bacteria and virulence factor. However, as the infected fish with lower concentrations of *A. hydrophila* showed no clinical signs and mortality, other relevant factors including the drastic environmental changes, improper management and secondary infections in fish farming may also cause outbreaks of bacterial diseases. Therefore, additional studies should be conducted in search for improvements in immunological resistance of African catfish against the pathogenicity of *A. hydrophila.*

Conflict of Interest

The authors declare that there is no conflict of interest that is related to the work.

Acknowledgment

We thank all reviewers for providing critical feedback and comments that greatly improved the manuscript.

Funding

This research was funded by the Ministry of Higher Education (MoHE), Malaysia under the Knowledge Transfer Programme (KTP) [FI-ECO/4 (UPM/13)].

Author Contribution

Conceptualization: Daud, H.M.
Data curation: Mohamad, N.F.A.
Methodology: Mohamad, N.F.A.
Formal analysis: Mohamad, N.F.A.
Visualisation: Mohamad, N.F.A.
Software: Mohamad, N.F.A.
Writing (original draft): Mohamad, N.F.A.
Writing (review and editing): Manaf, S.R.
Validation: Manaf, S.R.
Supervision: Daud, H.M.
Funding acquisition: Daud, H.M., & Manaf, S.R.
Project administration: Daud, H.M.

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