Detection of Virulence Genes in *Aeromonas hydrophila* Isolated from Hybrid Tilapia, *Oreochromis* spp.

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Detection of virulence genes in *Aeromonas hydrophila* isolated from freshwater fish

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_____

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Detection of virulence genes in *Aeromonas hydrophila* isolated from freshwater fish

This research paper, presented to the Faculty of Veterinary Medicine at Universiti Malaysia Kelantan, represents a crucial step in advancing our understanding of pathogenic microorganisms within aquatic environments. It fulfills a partial requirement for the course DVT 55204 - Research Project, which emphasizes the significance of research in veterinary medicine.

ABSTRACT

Aeromonas hydrophila, a pathogenic Gram-negative bacterium, poses significant risks to both aquatic animals and human health. It is well-documented for its role in causing a range of infections, from mild gastroenteritis in humans to severe systemic diseases in fish. Given its prevalence in freshwater ecosystems, understanding its pathogenicity is vital for developing effective management strategies in aquaculture and mitigating potential public health threats.

This study aimed to identify virulence genes present in various Aeromonas hydrophila samples isolated from hybrid tilapia. The samples analyzed included K3T11(1), K310, K3T8, K1T2(1), and K2T6(b). This selection highlights the diverse ecological niche that Aeromonas hydrophila occupies and its capacity to adapt to different host environments.

To achieve this objective, Polymerase Chain Reaction (PCR) was employed as a molecular technique to detect specific virulence genes associated with the pathogenicity

of Aeromonas hydrophila. The targeted virulence genes included Haemolysin (hlyA), Aerolysin (aerA), Cytolytic Enterotoxin (act), AscV, and AopB. These genes are crucial as they contribute to the bacterium's ability to cause disease by damaging host tissues, evading immune responses, and facilitating the establishment of infection.

The results of the PCR analysis were particularly revealing. Among the samples tested, only the K2T6(b) sample showed a positive result for the Cytolytic Enterotoxin gene (act). This finding is significant, as it indicates the presence of a key virulence factor that may enhance the pathogenic potential of the bacterium in that particular sample. The exclusive identification of this gene in the K2T6(b) sample suggests variability in virulence gene distribution among different Aeromonas hydrophila isolates, which could influence their respective pathogenicity.

These findings underscore the critical importance of monitoring virulence factors in Aeromonas hydrophila, as they have far-reaching implications for both aquaculture practices and public health initiatives. By identifying and understanding these virulence genes, we can better assess the risks posed by this pathogen in aquatic systems and develop targeted strategies for disease prevention and control in both fish populations and potentially affected human communities. The research contributes valuable insights to the field of veterinary medicine and highlights the need for ongoing surveillance and research into aquatic pathogens.

Keywords: *Aeromonas hydrophila*, virulence genes, polymerase chain reaction, sequencing,

ABSTRAK

Kertas penyelidikan ini, yang dibentangkan kepada Fakulti Perubatan Veterinar di Universiti Malaysia Kelantan, merupakan langkah penting dalam memajukan pemahaman kita tentang mikroorganisme patogen dalam persekitaran akuatik. Ia memenuhi syarat sebahagian untuk kursus DVT 55204 - Projek Penyelidikan, yang menekankan kepentingan penyelidikan dalam perubatan veterinar.

Fokus utama kajian ini adalah *Aeromonas hydrophila*, bakteria Gram-negatif patogen yang berisiko tinggi kepada kesihatan haiwan akuatik dan manusia. *Aeromonas hydrophila* telah didokumenkan dengan baik dalam peranannya menyebabkan pelbagai jangkitan, dari gastroenteritis ringan pada manusia hingga penyakit sistemik yang teruk dalam ikan. Memandangkan prevalensinya dalam ekosistem air tawar, memahami patogenisitasnya adalah penting untuk membangunkan strategi pengurusan yang berkesan dalam akuakultur dan mengurangkan ancaman kesihatan awam yang berpotensi. Objektif utama penyelidikan ini adalah untuk mengenal pasti gen virulensi yang terdapat dalam pelbagai sampel *Aeromonas hydrophila*. Sampel yang dianalisis dalam kajian ini termasuk K3T11(1), K310, K3T8, K1T2(1), dan K2TK(b), yang kesemuanya diasingkan dari ikan air tawar. Pemilihan ini menyoroti kepelbagaian niche ekologi yang diduduki oleh *Aeromonas hydrophila* dan keupayaannya untuk menyesuaikan diri dengan persekitaran hos yang berbeza. Untuk mencapai objektif ini, teknik Reaksi Rantai Polimerase (PCR) digunakan sebagai teknik molekul untuk mengesan gen virulensi tertentu yang berkaitan dengan patogenisitas *Aeromonas hydrophila*. Gen virulensi yang

disasarkan termasuk Haemolysin (hlyA), Aerolysin (aerA), Cytolytic Enterotoxin (act), AscV, dan AopB. Gen-gen ini adalah penting kerana ia menyumbang kepada kemampuan bakteria untuk menyebabkan penyakit dengan merosakkan tisu hos, mengelak respons imun, dan memudahkan penubuhan jangkitan. Hasil analisis PCR sangat menarik. Antara sampel yang diuji, hanya sampel K2T6(b) menunjukkan keputusan positif untuk gen Cytolytic Enterotoxin (act). Penemuan ini adalah penting, kerana ia menunjukkan kehadiran faktor virulensi utama yang mungkin meningkatkan potensi patogen bakteria dalam sampel tersebut. Pengenalan eksklusif gen ini dalam sampel K2T6(b) mencadangkan variasi dalam pengedaran gen virulensi di kalangan isolat *Aeromonas hydrophila* yang berbeza, yang mungkin mempengaruhi patogenesis masing-masing. Penemuan ini menekankan kepentingan kritikal untuk memantau faktor virulensi dalam *Aeromonas hydrophila* kerana ia mempunyai implikasi jauh untuk amalan akuakultur dan inisiatif kesihatan awam. Dengan mengenal pasti dan memahami gen virulensi ini, kita dapat menilai risiko yang ditimbulkan oleh patogen ini dalam sistem akuatik dan membangunkan strategi terarah untuk pencegahan dan kawalan penyakit dalam populasi ikan dan komuniti manusia yang berpotensi terjejas. Penyelidikan ini memberikan wawasan berharga kepada bidang perubatan veterinar dan menekankan perlunya pemantauan dan penyelidikan yang berterusan mengenai patogen akuatik.

ACKNOWLEDGEMENT

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List of abbreviations

Act - cytotoxic enterotoxin

AerA - Aerolysin

DNA - Deoxyribonucleic acid

HGT - Horizontal gene transfer

HlyA - Hemolysin

LPS - Lipopolysaccharides

MAS - Motile Aeromonas septicemia

NaCL - Sodium chloride

OMPs - Outer membrane proteins

PCR - Polymerase Chain Reaction

TSA - Tryptic Soy Agar

TSB - Trypticase Soy Broth

T3SS - Type III secretion system

CHAPTER 1

INTRODUCTION

Aeromonas hydrophila, a Gram-negative rod-shaped bacterium, exhibits versatility in oxygen levels and yields positive results for catalase, oxidase, and indole tests. Known for its role in primary and secondary infections within fish communities, it thrives in various aquatic habitats, including benthic sediments and the microflora of freshwater fish, notably within their skin mucus and digestive tract (Zmysłowska et al., 2009). This bacterial genus comprises three motile species: *A.hydrophila, A.caviae, and A.sobria*. Identified as an opportunistic pathogen, *A. hydrophila* has been associated with significant mortality in both farmed and wild fish populations (Harikrishnan and Balasundaram, 2005). According to Rodrigues et al. (2019), *A. hydrophila* has been implicated in outbreaks in fish farms, resulting in substantial economic losses to the aquaculture industry worldwide. Semwal et al. (2023) further highlight the role of motile Aeromonas species, particularly *A. hydrophila*, as key agents causing various infections, with Aeromonas being the most prevalent bacterial disease year-round in Indian major carp and exotic carp. Among tested fish species, *H.molitrix* shows increased susceptibility to Aeromonas. Ahangarzadeh et al. (2020) delve into the complex pathogenesis of *Aeromonas hydrophila* infections, involving numerous virulence factors such as lipopolysaccharides, outer membrane proteins, pili, flagella, the type III secretion system (T3SS), along with various extracellular enzymes and toxins. This ongoing investigation aims to identify specific virulence genes, including hemolysin, aerolysin,

cytolytic enterotoxin, and components of the T3SS (aopB and ascV), utilizing molecular techniques, particularly polymerase chain reaction (PCR).

1.1 RESEARCH PROBLEM STATEMENT

The existence of pathogenic components within *Aeromonas hydrophila* substantially impacts the economic stability of freshwater fish farming. According to Ahmed et al. (2018), *Aeromonas hydrophila* is a zoonotic disease caused by consuming contaminated fish, seafood, and drinking water or direct contact with recreational water sources. Additionally, differences in virulence gene profiles between *Aeromonas hydrophila* isolates need to be elucidated to understand how these bacteria adapt to different environments and hosts.

1.2 RESEARCH QUESTION

Which virulence genes can be detected in *Aeromonas hydrophila* isolated from hybrid tilapia?

1.3 RESEARCH HYPOTHESIS

Virulence genes such as hemolysin, aerolysin cytolytic enterotoxin, and T3SS (*aop*B and *asc*V) can be detected from *Aeromonas hydrophila* isolated from hybrid tilapia.

1.4 RESEARCH OBJECTIVES

To identify the virulence genes in *Aeromonas hydrophila* obtained from hybrid tilapia

CHAPTER 2

LITERATURE REVIEW

2.2 Global economic impact due to *Aeromonas hydrophila*

The economic impact of issues related to *Aeromonas hydrophila* spans various sectors, particularly aquaculture and related industries. In Southeast Asia, the significant financial consequences of *A.hydrophila*-induced fish mortality are evident in the fish farming sector(Harikrishnan and Balasundaram, 2005). Saleh et al. (2021) documented that *A.hydrophila*, identified as one of the most aggressive Aeromonas species, presents incurable challenges to freshwater fish aquaculture in Egypt. In the period from June to October 2009, an outbreak affected 48 catfish farms in west Alabama, USA, resulting in substantial losses exceeding 3 million pounds (approximately 1339 metric tons) of food-size channel catfish (Ictalurus Punctatus) (Pridgeon and Klesius, 2011). Recent data from the Department of Fisheries, as cited by Anjur et al. (2021), reveal that Malaysia ranks as the 8th largest producer of ornamental fish globally, with over 70% of these species exported. However, the aquaculture sector encounters significant challenges, including disease outbreaks, leading to considerable economic losses and potentially threatening industry sustainability.

2.2 *Aeromonas hydrophila* in aquaculture and human

Aeromonas hydrophila poses a significant threat to both aquaculture and human health, with documented associations with food and waterborne illnesses, particularly in developing nations where hygiene and water quality are subpar (Rodrigues et al., 2019). This pathogenic organism is frequently linked to disease outbreaks in warm water fish farming operations worldwide and is recognized as an opportunistic pathogen capable of causing a range of ailments in aquatic creatures, such as abdominal swelling, skin inflammation, and bloodstream infections (Abreu et al., 2017; Anjur et al., 2021). Additionally, *Aeromonas hydrophila* can lead to motile Aeromonas septicemia (MAS), characterized by symptoms like bleeding, lesions, and fluid accumulation in the abdomen, resulting in increased mortality rates and economic burdens for aquaculture practitioners (Anjur et al., 2021). In humans, these microorganisms often cause gastrointestinal illnesses that, if left untreated, can progress to systemic infection, including septicemia (Pessoa et al., 2019). Moreover, *Aeromonas hydrophila* can invade various bodily tissues, causing ailments such as eye, respiratory, and joint infections, as well as bone infections, often originating from previous instances of bloodstream infection.

2.3 Virulence genes in *Aeromonas hydrophila*

Several studies have investigated the presence of virulence genes in *Aeromonas hydrophila*, revealing its capacity to induce various infections through a complex pathogenic process involving proteic toxins such as hemolysin, aerolysin, cytotoxin, enterotoxin, hemagglutinin, surface array proteins, and enzymes like protease and elastase (Ahangarzadeh et al., 2020). Nhinh et al. (2021) found that aerolysin (aerA) and cytotoxic enterotoxin (act) were frequently identified among *A.hydrophila* isolates from freshwater fish species. Similarly, Rogers et al. (2020) observed hlyA, aerA, and exu as the most prevalent virulence genes, with exu being present in 63.7% of the isolates. In their research, Omar and Zayed (2016) used PCRto examine three *A.hydrophila* strains for the presence of five virulence genes, revealing that two strains harbored all five genes (ast, act, hlyA, aer, and alt), while the third strain possessed four genes(ast, act, hlyA, and aer). Pattanayak et al. (2020) investigated the presence and alteration of various virulence genes (aerolysin, hemolysin, cytoen, amp, elastase, flagellin, lipase, βhemolysin, and T3SS) in seven *A.hydrophila* isolates under different temperature conditions in vivo system.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Bacterial collection

Aeromonas hydrophila isolates were obtained from archived samples stored in the Trypticase Soy Broth(TSB) added with 50% glycerol stocks at -80˚C in the freezer in Zoonotic Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The isolates were revived in Tryptic Soy Agar(TSA) and incubated at 30˚C for 24 hours.

Table 1: The list of *Aeromonas hydrophila* isolates

No	**Bacterial isolates**	**Sampling site**
1.	K3T11(1)	Kg.Tujuh,Tumpat,Kelantan
2.	K3T10	Kg.Tujuh,Tumpat,Kelantan
3.	K3T8	Kg.Tujuh,Tumpat,Kelantan
4.	K1T2(1)	Kg.Tujuh,Tumpat,Kelantan
5.	K2T6(b)	Kg.Pantai Melawi, Bachok,Kelantan

3.2 Extraction of DNA by boiling method

The boiling method, with modifications based on Dashti et al. (2009), was employed in this study. A single bacterial colony was suspended in 1 mL of 0.85% NaCl within a 1.5

mL centrifuge tube. The tube was centrifuged at 12,000 rpm for 5 minutes, and the supernatant was discarded. The resulting pellet was resuspended in 500 µL of sterile distilled water and thoroughly vortexed. The bacterial suspension was then incubated in a water bath at 95°C for 15 minutes, followed by immediate cooling on ice for 15 minutes. Afterward, the suspension was centrifuged at 12,000 rpm for 5 minutes, and the clear supernatant was transferred to a new 1.5 mL centrifuge tube for storage at -20°C. The sample was centrifuged again to sediment any remaining debris, and the supernatant was collected.

3.3 Polymerase Chain Reaction(PCR)

The primers utilized in this study to detect the presence of virulence genes were selected based on previous research. A total of nine primers were used, each with a specific PCR protocol as detailed in Table 2. The reagents and volumes for a single PCR reaction, performed using the T100 Thermal Cycler (Bio-Rad, USA), included 12.5 µL of master mix (containing PCR buffer, Mg^{2+}, Cl^-, dNTPs, and Taq polymerase), 1 µL of forward primer, 1 µL of reverse primer, 8.5 µL of nuclease-free water, and 2 µL of DNA template. The primers for detecting virulence genes were listed in Table 2, with each primer following its specific protocol as referenced.

Table 2: Primers used for the amplification of virulence genes of Aeromonas hydrophila

Virulence genes	Primers	DNA sequences (5'-3')	Protocol	Product size	Reference
Haemolysin (*hly*A)	F	GGC CGG TGG CCC GAA GAT GCA GG	94°C for 5 min, 30 cycles at 94°C 30 sec, 68°C 30 sec, 72°C 2 min, and 72°C 5 min.	597	Granum et al. (1998)
	R	GGC GGC GCC GGA CGA GAC GGG			
Aerolysin (*aer*A)	F	GCA GAA CCC ATC TAT CCA G	94°C for 5 min, 30 cycles at 94°C 30 sec, 55°C 30 sec, 72°C 30 sec, and 72°C 5 min.	252	Dorsch et al. (1994)
	R	TTT CTC CGG TAA CAG GAT TG			

Cytolytic enterotoxin (*act*)	F	ATG ACC CAG TCC TGG CAC GG	94°C for 5 min, 30 cycles at 94°C 30 sec, 58°C 30 sec, 72°C 30 sec, and 72°C 5 min.	482	Ahangarzadeh et al. (2022)
	R	GCC GCT CAG GGC GAA GCC GC			
*Asc*V	F	GCGAGAAT GTTGTTG CCGTT	95°C for 5 min, 30 cycles at 94°C 60 sec, 58.5°C 50 sec, 72°C 60 sec, and 72°C 5 min.	137	Akmal et al. (2020)
	R	AACATGCG TGCGATT CTGGA			
*Aop*B	F	TCCAGCAA GTTCGCCT GTTT	95°C for 5 min, 30 cycles at 95°C 60 sec, 58.5°C 50	129	Akmal et al. (2020)
	R	CGCCATGA AAGCCTC			

		AAAT	sec, 72°C 60 sec, and 72°C 5 min.		

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3.4 Agarose Gel Electrophoresis

The amplified PCR products were analyzed using a 1.5% agarose gel stained with Midori Green. Electrophoresis was conducted at 100 V and 400 mA for 45 minutes. The gel was then photographed using a gel documentation system equipped with a UV transilluminator (Bio-Rad, USA).

3.5 Sequencing and Analysis

PCR products were sent to Apical Sdn. Bhd. for further sequencing. The sequence information then was compared with Genbank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

CHAPTER 4

RESULTS

In this study, among the samples used, such as K3T11(1), K310, K3T8, K1T2(1), and K2T6(b) isolated, only K2T6(b) showed the Cytolytic Enterotoxin (act) gene, highlighting variability in virulence among isolates. To further confirm this result, the gene was reamplified using Polymerase Chain Reaction (PCR), producing a prominent band. For further confirmation, the isolate K2T6(b) was sent for sequencing. The results identified the isolate as *Plesiomonas shigelloides*. The sequence information was then compared with GenBank using BLAST to validate the findings.

CHAPTER 5

DISCUSSION

This study assessed the presence of key virulence genes in *Aeromonas hydrophila* isolated from hybrid tilapia. Among the five isolates analyzed, only one carried the *act* gene, encoding cytolytic enterotoxin, while no isolates tested positive for *hlyA*, *aerA*, *AscV*, or *AopB*.

The detection of the *act* gene in a single isolate is significant. This gene is known to encode a toxin that contributes to cellular lysis and disruption of intestinal barriers in host organisms. Cytolytic enterotoxins are major contributors to the pathogenicity of *A. hydrophila*, associated with diseases such as hemorrhagic septicemia in fish and gastroenteritis in humans (Hossain et al., 2021; Zhang et al., 2023). However, the absence of other key virulence genes, such as *hlyA* and *aerA*, suggests that the pathogenic potential of these isolates may be limited or strain-specific. Studies have shown that the presence and expression of virulence genes in *A. hydrophila* are highly variable, and influenced by genetic diversity and environmental factors (Tekedar et al., 2021; Kim et al., 2023).

In contrast to clinical isolates, which often harbor multiple virulence factors, environmental strains of *A. hydrophila* tend to exhibit lower virulence gene profiles. For example, Zhang et al. (2020) reported that clinical isolates from infected humans frequently carry combinations of *act*, *hlyA*, and *aerA*, whereas aquatic isolates often lack one or more of these genes. This suggests a differential adaptation of *A. hydrophila* based on its ecological niche.

Environmental conditions, such as water quality, temperature, and nutrient levels, play a crucial role in shaping the virulence profiles of *A. hydrophila*. Stressful environments, including polluted or nutrient-rich waters, have been shown to upregulate virulence genes, enabling the bacterium to exploit weakened host defenses (Rao et al., 2022). Conversely, the absence of such stressors may reduce selective pressure for maintaining these genes, as observed in this study.

The absence of *hlyA* and *aerA* in our isolates contrasts with studies from heavily polluted water systems, where these genes are prevalent. Tekedar et al. (2021) and Yadav et al. (2019) found that these hemolysins, which contribute to host cell lysis and biofilm formation, were more common in isolates from contaminated environments. This difference highlights the need to consider the ecological context when interpreting virulence gene prevalence.

Horizontal gene transfer (HGT) is a critical mechanism influencing the distribution of virulence factors among *A. hydrophila* strains. Genes encoding toxins, hemolysins, and secretion systems can be acquired through mobile genetic elements such as plasmids and transposons (Kim et al., 2023). The sporadic presence of the *act* gene in our isolates may reflect recent HGT events or the loss of other virulence factors over time due to reduced selective pressure. Zhang et al. (2023) demonstrated that HGT plays a pivotal role in the dissemination of virulence determinants in aquatic pathogens, especially in regions with high bacterial density and genetic exchange.

The absence of *AscV* and *AopB*, key components of the type III secretion system (T3SS), in all isolates is noteworthy. The T3SS is a specialized structure used by many gram-negative bacteria, including *A. hydrophila*, to inject effector proteins into host cells, facilitating immune evasion and tissue colonization (Rao et al., 2022). Zhao et al. (2024) observed that the loss of T3SS-associated genes can significantly reduce the virulence of *A. hydrophila*, as these systems are essential for host-pathogen interactions. The absence of T3SS genes in our isolates may indicate reduced pathogenicity in fish or adaptation to environmental survival rather than active infection.

The identification of the *act* gene in one isolate has significant implications for aquaculture and public health. While this study indicates a relatively low virulence potential overall, the presence of this gene highlights the risk of opportunistic infections in stressed or immunocompromised fish. In aquaculture, such infections can lead to economic losses and may also pose a zoonotic risk through the consumption of contaminated fish or water (Hossain et al., 2021). Preventative measures, such as maintaining water quality and reducing fish stress, are essential to minimize these risks.

While this study provides valuable insights, it is limited by its small sample size and the focus on a narrow set of virulence genes. Recent studies have highlighted the importance of other virulence factors, such as lipopolysaccharides (LPS), outer membrane proteins (OMPs), and exoproteins, which were not examined in this study (Hossain et al., 2021; Zhao et al., 2024). Furthermore, whole-genome sequencing of transcriptome analyses could reveal additional insights into the virulence mechanisms and adaptive strategies of *A. hydrophila* in freshwater environments. Future research should focus on larger sample sizes and include isolates from diverse geographical and ecological settings to better understand the distribution of virulence factors. The role of environmental stressors in modulating virulence gene expression also warrants further investigation. Finally, in vivo studies using fish models could provide direct evidence of the pathogenic potential of *act*-positive isolates.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

This study investigated the prevalence of virulence genes (*act*, *hlyA*, *aerA*, *AscV*, and *AopB*) in *Aeromonas hydrophila* isolated from freshwater fish. Among the five isolates tested, only the *act* gene was detected, while the other virulence genes were absent. The *act* gene, known to encode a cytolytic enterotoxin, plays a significant role in tissue damage and the disruption of host barriers, highlighting the pathogenic potential of this isolate. However, the absence of *hlyA*, *aerA*, and type III secretion system genes (*AscV* and *AopB*) in all isolates suggests limited overall virulence in the studied strains. These findings align with observations from other studies, where environmental strains of *A. hydrophila* are generally less virulent than clinical isolates. The variability in virulence gene profiles reflects the bacterium's ecological adaptability, which is influenced by factors such as water quality, nutrient availability, and host interactions. This research contributes valuable insights into the pathogenicity of *A. hydrophila* in aquaculture, underscoring the need for vigilant monitoring to prevent disease outbreaks and safeguard both fish and human health.

To mitigate the risks associated with *A. hydrophila*, several actions are recommended. Regular monitoring of aquaculture environments should be conducted to track the presence of virulence genes and assess potential risks. Diagnostic techniques should be enhanced through the use of advanced genomic tools such as whole-genome sequencing

and transcriptomics, which can provide deeper insights into the genetic diversity and regulatory mechanisms of this bacterium. Maintaining optimal water quality in aquaculture systems is crucial for reducing stress on fish and preventing opportunistic infections. Additionally, promoting public awareness about proper handling, preparation, and cooking of freshwater fish is essential to minimize the risk of zoonotic transmission of *A. hydrophila* to humans.

Future studies should include larger sample sizes and isolates from diverse geographical and ecological settings to better understand the global distribution of virulence genes in *A. hydrophila*. Expanding the scope of research to include additional virulence factors, such as lipopolysaccharides (LPS) and outer membrane proteins (OMPs), could provide a more comprehensive view of the bacterium's pathogenicity. Moreover, in vivo studies using fish models would offer direct evidence of the pathogenic potential of *act*-positive isolates and allow for the evaluation of intervention strategies. By addressing these research gaps and implementing effective management practices, the aquaculture industry and public health sectors can better control the risks associated with *A. hydrophila*, ensuring the sustainability of fish farming and the safety of fish-derived products for human consumption.

Appendices

Figure 1: Preparation of nutrient agar and broth

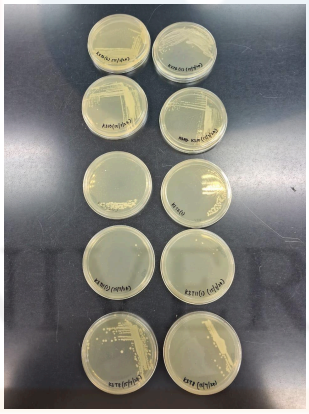

Figure 2: Bacterial isolation

Figure 3: Bacterial isolation on blood agar

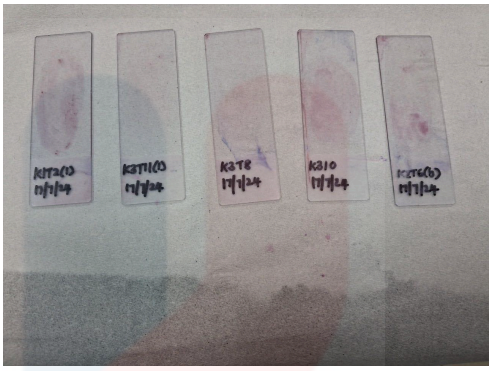

Figure 4: Gram staining

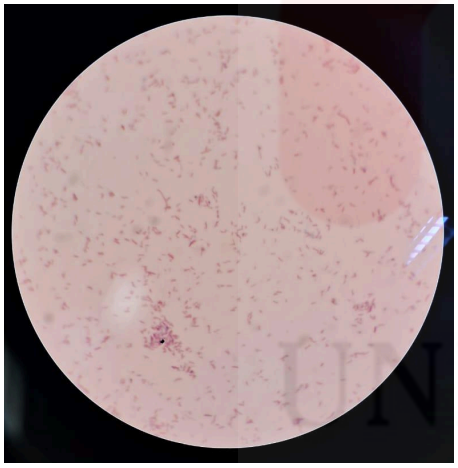

Figure 5: Bacterial identification

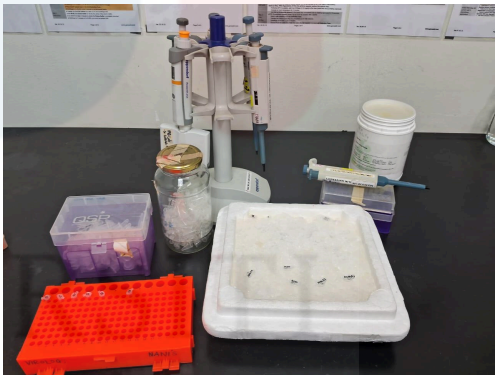

Figure 6: DNA extraction

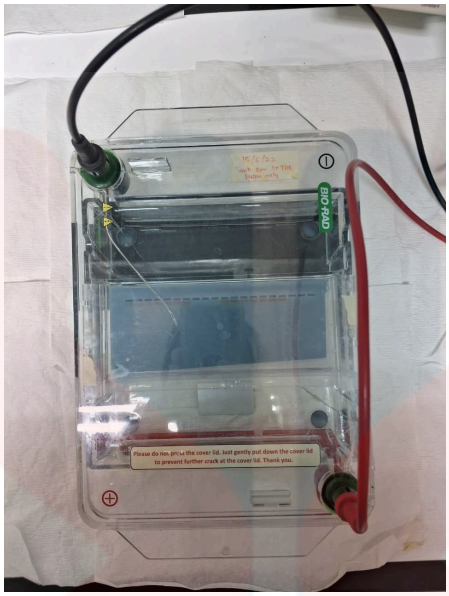

Figure 7: Agarose Gel electrophoresis

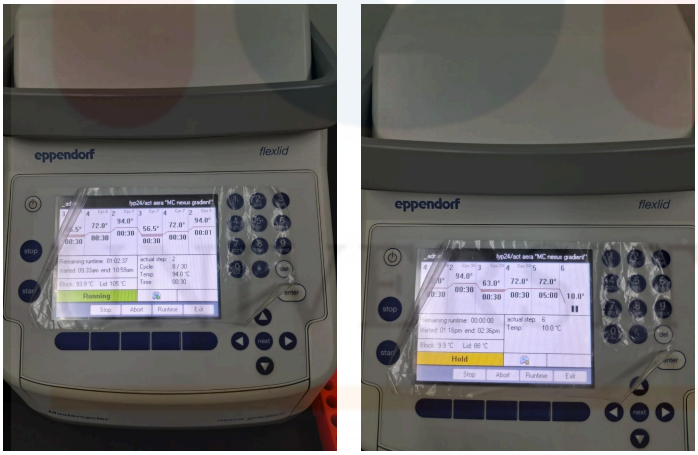

Figure 8: Polymerase Chain Reaction(PCR)

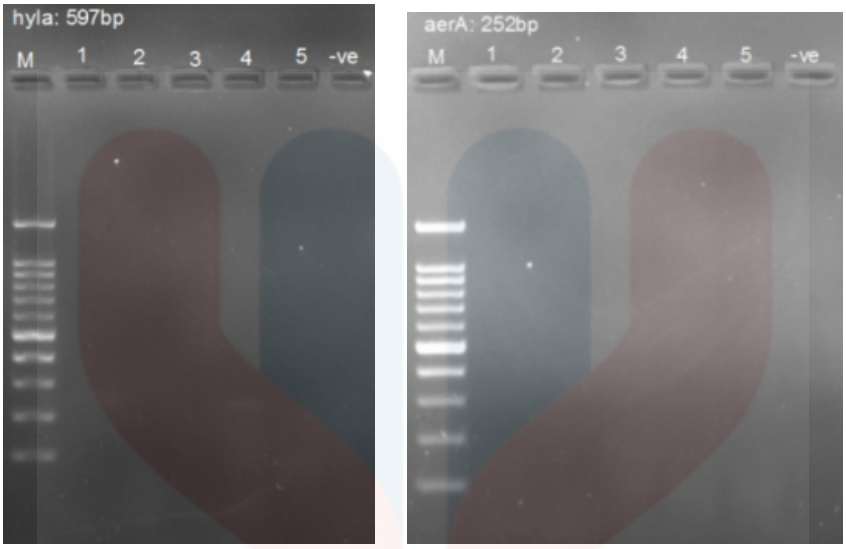

Figure 9: PCR results of hyla and aerA

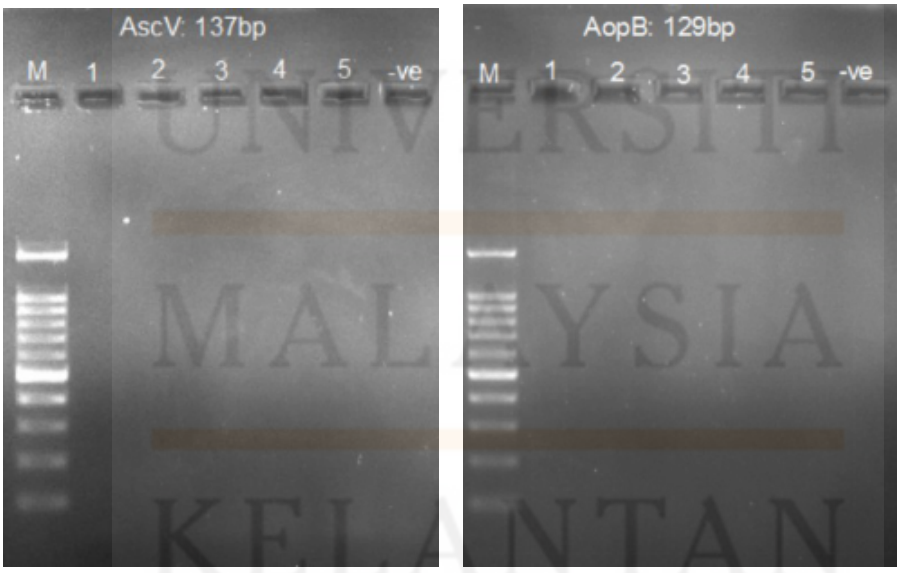

Figure 10: PCR results of ascV and aopB

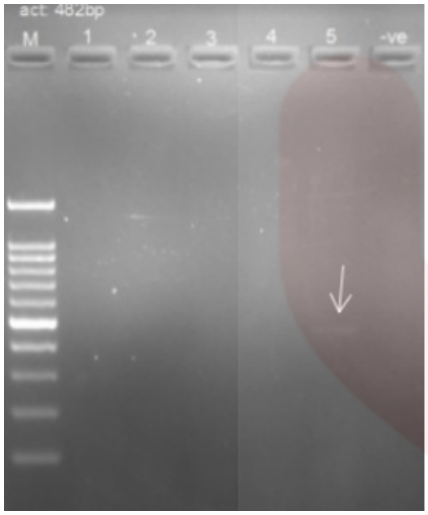

Figure 11: PCR result of act act

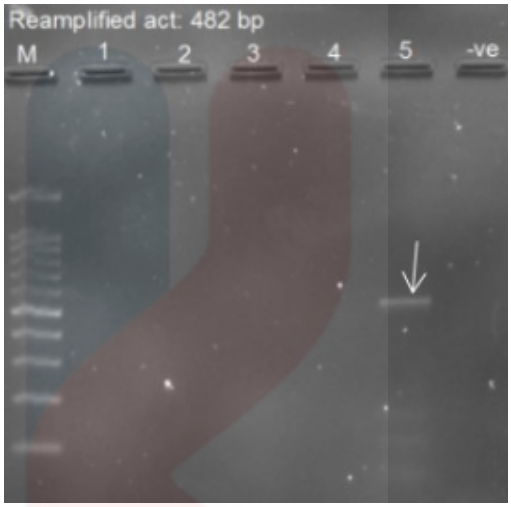

Figure 12: PCR result of reamplified

Figure 13: With lab assistant Kak Salma

Figure 14: With supervisor Dr.Ruhil

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