Malaysian Applied Biology
https://doi.org/10.55230/mabjournal.v54i3.3424

Research Article

Discovery of *Aeromonas hydrophila* in the Sea Surface Microlayer at Pulau Tenggol, Terengganu

Moh Yuen Mun[1], Nik Nuraznida Nik Ibrahim[1], Wan Syaidatul Aqma[1]*

1. Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
*Corresponding author:syaidatul@ukm.edu.my

ABSTRACT

Aeromonas hydrophila is a formidable opportunistic pathogen that represents a significant threat to both aquatic organisms and human health. Its presence in the sea surface microlayer (SML) poses a potential risk to marine fauna, local communities, and visitors. To date, research on the abundance of bacterioneuston, particularly *A. hydrophila*, within the SML remains limited, as studies in Malaysia have primarily focused on physicochemical parameters and surfactant concentrations. This study was conducted to identify and characterize *A. hydrophila* isolated from the SML at Pulau Tenggol, Terengganu. The two *A. hydrophila* isolates identified demonstrated notable pathogenic potential, as evidenced by positive reactions in all virulence factor tests and strong biofilm-forming capabilities. Alarmingly, they were found to be susceptible only to chloramphenicol, raising concerns about antibiotic resistance. *A. hydrophila* is naturally found in seawater; therefore, the relatively low abundance observed in this study does not constitute an immediate public health concern, as at low concentrations it is generally regarded as posing minimal risk to healthy populations. However, even low levels of *A. hydrophila* in water can still cause disease, particularly in immunocompromised individuals. Therefore, the pathogenic potential of the detected strains, coupled with the likelihood of bacterial contamination, highlights the importance of regular monitoring and stringent quality control measures for the SML to mitigate potential risks to the ecosystem and human health.

Key words: *Aeromonas hydrophila*, pathogen, sea surface microlayer, virulence factor

INTRODUCTION

The sea surface microlayer (SML) is a highly dynamic zone, serving as a vital boundary between the atmosphere and the ocean. It plays a central role in climate regulation, nutrient cycling, and the exchange of energy and materials between the ocean and the atmosphere, maintaining the delicate balance of our planet's environmental systems (Engel *et al*., 2017; Wurl *et al*., 2017). Due to the complex interactions between the hydrosphere and atmosphere, the conditions in the SML are often unstable and subject to rapid changes. The SML around Malaysia is particularly unique, as it is also affected by the monsoons and tropical climate. This instability can present challenges for marine organisms and impact the life forms within the SML, particularly microorganisms, as the fluctuations in conditions directly influence their survival (Skórczewski & Mudryk, 2009; Wurl *et al*., 2019).

Most microorganisms inhabiting the SML are bacteria, protists, and plankton—collectively known as neuston—with bacteria specifically referred to as bacterioneuston. These microbial communities differ significantly from those in the underlying water column or other marine habitats, both in taxonomic composition and ecological function. Moderate concentrations of certain chemical compounds can be beneficial to microorganisms in the SML; however, human activities such as oil spills and industrial discharges have introduced heavy metals, synthetic chemicals, and antibiotics into this layer. When these pollutants exceed optimal concentrations, they can exert severe ecotoxicological effects on bacterioneuston (Wurl *et al*., 2011; Wurl *et al*., 2019). Benhalima *et al*. (2023) reported that *A. hydrophila* tolerates heavy metals, some of which function as essential micronutrients for enzymatic processes—for example, copper needed for enzymes involved in several metabolic activities, especially those linked to microbial respiration, oxidative stress defense, and nutrient cycling (Argüello *et al*., 2013).

The enrichment of such metals in polluted SML environments may enhance the metabolic capacity and survival of *A. hydrophila* under conditions where other species cannot persist. Prolonged exposure to these environmental stressors has driven adaptive responses in bacterioneuston, increasing their resistance to multiple stress factors. However, this resilience also presents a public health concern, as highly resistant bacteria capable of surviving the dynamic conditions of the SML are often pathogenic. Consequently, the SML serves as an ecological niche for specialized bacterial populations, indirectly acting as a reservoir for potential pathogens (Prabhakaran *et al*., 2016; Michaud *et al*., 2018; Abdool-Ghany *et al*., 2023). At present, global investigations into the abundance of bacterioneuston, particularly pathogenic taxa and the associated community interactions within the SML, are scarce. In Malaysia, existing research on the SML has been largely confined to assessments of physicochemical properties and surfactant concentrations. (Latif & Mustaffa, 2013; Mustaffa *et al*., 2013; Jaafar *et al*., 2014; Jaeger *et al*., 2019; Uning *et al*., 2022).

Article History
Accepted: 21 August 2025
First version online: 30 September 2025
Cite This Article:
Mun, M.Y., Nik Ibrahim, N.N. & Aqma, W.S. 2025. Discovery of *Aeromonas hydrophila* in the sea surface microlayer at Pulau Tenggol, Terengganu. Malaysian Applied Biology, 54(3): 135-148. https://doi.org/10.55230/mabjournal.v54i3.3424

A. hydrophila demonstrates a remarkable ability to survive in a wide range of environmental conditions and is frequently found in habitats characterised by hot seasons or warm climates (Guz & Sopińska, 2008; Sarkar & Rashid, 2012). Therefore, SML with high nutrient concentrations and warm environmental temperatures fostered the proliferation and survival of *A. hydrophila* (Djuikom *et al*., 2008). Its survival and pathogenicity are driven by key virulence factors, including flagellum, pili, fimbriae, secretion systems, quorum-sensing systems, and a protective capsule. This bacteria also produces exotoxins (aerolysin, cytolytic enterotoxin & hemolysin) and enzymes (lipase, proteases & nuclease) that damage host tissues and promote infection. (Janda & Abbott 2010; Oliveira *et al*., 2012; Rasmussen-Ivey *et al*., 2016; Awan *et al*., 2018; Fernández-Bravo & Figueras, 2020; Pauzi *et al*., 2020). Many researchers have linked disease outbreaks in aquatic animals to the virulence and toxicity of *A. hydrophila*, particularly its production of potent toxins such as aerolysin and hemolysin β (Laith & Najiah, 2014; Ahangarzadeh *et al*., 2022).

The presence of *A. hydrophila* in seafood and animal-based food products has also raised concerns regarding public health safety, which can result from improper food handling practices or contact with contaminated water sources or tainted food; additionally, its presence in drinking water and water used for daily purposes can lead to waterborne diseases (Chauret *et al*., 2001; Daskalov, 2006; Janda & Abbott, 2010; Fernández-Bravo & Figueras, 2020). Current global trends indicate that *A. hydrophila* infections are no longer limited to enteric diseases, and therefore, the country's treatment methods and standard operating procedures must be adapted to ensure the safety of public health. Monitoring the management system for the cleanliness and quality of water resources is essential to ensure the health and safety of both animals and humans. Early detection of pathogens can minimise the risk of exposure and the transmission of diseases. The water samples of SML at Tenggol Island were selected in this study due to Pulau Tenggol's reliance on water-based tourism and ocean-based industries (such as diving and fishing), emphasizing the significance of public health safety. Hence, this study was carried out to investigate the presence of *Aeromonas hydrophila* in the SML at Pulau Tenggol, Terengganu, and to explore its characteristics.

MATERIALS AND METHODS

Sampling

The SML samples were collected using the glass plate technique (Harvey & Burzell, 1972) from coastal seawater at Pulau Tenggol, Terengganu, in July, at the coordinates latitude 4°48'28"N and longitude 103°43'4.8"E. Five replicate samples were obtained from a boat under calm and stable water conditions. The SML water was sampled at a thickness of ≤1 µm. All sampling tools were sterilized and cleaned before and between samplings. Physical parameters of the seawater at the sampling site, including temperature (°C), dissolved oxygen (DO, mg L^{-1}), salinity (ppt), and pH, were measured using a YSI 556 MPS (USA) multiparameter probe. After collection, the water samples were stored in a cold box with ice to preserve sample quality and transported to the laboratory for processing within a day.

Bacteria isolation

Initially, a 0.1 mL aliquot of the water sample was streaked onto nutrient agar to assess the total bacterial count (CFU/mL). For bacterial isolation purposes, five sets of saline solution, 9 mL (0.85% sodium chloride, NaCl) were prepared in advance in each universal tube for serial dilution, ranging from 10^{-1} to 10^{-5}. A 0.1 mL aliquot from each dilution tube (10^{-1} to 10^{-5}) was pipetted onto nutrient agar and modified Rimler Shotts selective agar (mRS; pH 7.8) [Materials and chemicals that needed to produce mRS agar: 5.0 g of L-lysine HCl, 6.5 g of L-ornithine HCl, 0.3 g of L-cysteine HCl, 7.5 g of maltose, 6.8 g of sodium thiosulfate, 0.03 g of bromothymol blue, 0.8 g of ferric ammonium chloride, 5.0 g of bile salts, 3.0 g of yeast extract, 13.0 g of Bacto agar powder and 1 L of distilled water.] and spread using the spread plate technique. The plates were then incubated at 30°C for 24 hr. Cream-white coloured single colonies from nutrient agar were randomly selected, while yellow, round, circular colonies observed on mRS agar were isolated. All of the isolated bacterial colonies were subcultured multiple times using the streak plate technique on nutrient agar (containing 0.85% NaCl) to obtain pure colonies for further characterisation and identification of bacterial species.

Preliminary identification with culture method

The initial identification of the isolates was based on growth on mRS selective agar, as well as a series of biochemical tests. These tests included the Gram staining, catalase test, oxidase test, motility test, sulfur and indole production, Voges-Proskauer methyl red test, citrate test, triple sugar iron test, and the Vibriostatic disk O/129 test, as suggested by Nahar *et al*. (2016) and Ahmad (2001). *Aeromonas hydrophila* served as the positive control for each test, while *Escherichia coli* was used as the negative control. The bacterial strains used in this study were obtained from the culture collection of the Marine Biotechnology and Microbiology Laboratory, National University of Malaysia.

Virulence factor test

All isolates exhibiting characteristics consistent with positive control *A. hydrophila* based on the biochemical test results were identified as *A. hydrophila*. All the identified A.hydrophila were cultured on the nutrient agar and incubated at 30°C for 24 hr to obtain pure isolates. These isolates were subsequently tested for the presence of virulence factors, including protease, lipase, hemolysin, amylase, and DNase activity, as described by Ahmad (2001) and Chenia and Duma (2017).

Biofilm formation test

Biofilm formation was determined using a biofilm assay conducted in 96-well microtiter plates, based on the method described by Stepanović *et al*. (2007). All *Aeromonas spp*. Bacterial isolates used in the biofilm formation assay were grown in marine broth and incubated overnight at 30°C. Each bacterial culture was then adjusted to achieve an optical density reading between 0.8 and 1.0 at 590 nm absorbance. Next, 200 µL of bacterial suspension was added to each well of the 96-well microtiter plate in triplicate. For controls, 200 µL of sterile marine broth was used as a negative control. The microtiter plate was covered with a

sterile aluminium lid and incubated at 30°C for 24 hr.

After incubation, each well was rinsed two to three times with distilled water to remove non-adherent bacterial cells. Then, 200 µL of 0.1% crystal violet dye was added to each well and left for 20 min at room temperature. Following this, the stained wells were rinsed with distilled water to remove excess crystal violet. The microtiter plate was left to dry, and then 200 µL of 99% ethanol was added to dissolve the crystal violet from the well surfaces. The absorbance of the solution was measured at 595 nm using a microtiter plate reader (Bio-Rad model 680). The biofilm formation ability of the bacterial isolates was interpreted based on the standards as follows (Stepanović *et al*., 2007): ODs ≤ ODc, non-biofilm producer; ODc < ODs ≤ 2 ODc, weak biofilm producer; 2 ODc < ODs ≤ 4 ODc, moderate biofilm producer; 4 ODc < ODs, strong biofilm producer.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Mueller-Hinton agar (MHA; Oxoid) disk diffusion method with antibiotic-containing disks. *A. hydrophila* were tested against several antibiotics, including colistin sulfate (10 µg), ampicillin (10 µg), oxytetracycline (30 µg), penicillin (110 µg), polymyxin B (300 µg), chloramphenicol (10 µg), erythromycin (15 µg), nalidixic acid (30 µg), and sulfamethoxazole (25 µg). The *A. hydrophila* isolates were streaked on marine agar and incubated for 18–24 hr at 30°C to obtain pure single colonies. Five colonies, each approximately 1 mm in size, were transferred to 5 mL of Mueller-Hinton broth. A sterile cotton swab was dipped in the broth for 15 min, and the swab was then used to inoculate the MHA plate, ensuring even distribution. After 5 min, antibiotic disks were placed onto the surface of the agar plate. The plate was then incubated at 30°C for 24 hr, allowing the antibiotic to diffuse.

The diameter of the zone of inhibition around each antibiotic disk was measured and categorized according to the following scale: sensitive (S) ≥18 mm, intermediate (I) = 13–17 mm, and resistant (R) <13 mm (Thenmozhi *et al*., 2014). The MAR index was used as a tool to assess the risk of the isolates originating from regions with high or low antibiotic use. A MAR index >0.2 indicates a "high-risk" source of contamination.

Identification of *A. hydrophila* with PCR

Bacterial isolates that exhibited characteristics similar to *A. hydrophila* based on biochemical tests and virulence factor assessments were selected for identification via 16S rRNA primer sequencing. This method involves several key steps: DNA extraction from the bacterial isolate, PCR amplification using universal primer sets, analysis of PCR products via electrophoresis, and submission of the PCR product (amplified DNA from universal primer sets) to First Base Laboratory Sdn. Bhd. for sequencing, and finally, comparison of the obtained bacterial DNA sequence with sequences in the GenBank database.

DNA extraction

The *A. hydrophila* colonies were streaked onto marine agar and incubated overnight at 30°C to isolate pure colonies. All subsequent DNA extraction steps were performed at room temperature. Two to three pure colonies from the Petri dish were carefully transferred into an Eppendorf tube containing 1 mL of distilled water. The tube was then centrifuged for 2 min at 141.67 rpm in a microcentrifuge. After discarding the supernatant, the pellet was gently resuspended in 567 µL of TE buffer (10 mM Tris-HCl, pH 7.5; 10 mM EDTA). To initiate cell lysis, 30 µL of 10% sodium dodecyl sulfate (SDS) and 3 µL of Proteinase K were added, followed by incubation at 37°C for 1 hr. Subsequently, 100 µL of 5 M NaCl solution and 80 µL of hexadecyltrimethylammonium bromide (CTAB/NaCl) solution were introduced, and the mixture was incubated at 65°C for 10 min. Next, 0.7 mL of chloroform: isoamyl alcohol (24:1) solution was added, and the tube was centrifuged at 13,000 rpm for 5 min to remove the CTAB-protein complex and any residual polysaccharides. The aqueous phase (top layer) was carefully transferred into a new 1.5 mL Eppendorf tube. To further purify the sample, phenol: chloroform: isoamyl alcohol (25:24:1) solution was added in an equal volume to the transferred aqueous phase. After thorough mixing, the suspension was centrifuged at 13,000 rpm for 5 min, and the aqueous phase was transferred again into a fresh 1.5 mL Eppendorf tube. This extraction step was repeated until no protein was observed at the interphase. To precipitate the nucleic acids, 0.6 volumes of isopropanol (relative to the aqueous phase) were added to the solution. The tube was gently inverted several times before being centrifuged at 13,000 rpm for 20 min, resulting in the formation of a DNA pellet. The isopropanol was carefully discarded, and the DNA pellet was washed by adding 400 µL of 70% ethanol to the tube. After a final centrifugation at 13,000 rpm for 5 min, the supernatant was discarded, leaving the DNA pellet adhering to the tube wall. The pellet was allowed to air dry at room temperature. Finally, 50 µL of TE buffer was added to resuspend the DNA, and the sample was stored at -20°C for future analysis.

PCR and electrophoresis gel

Gene amplification was carried out using PCR with 16S rRNA universal primer set: forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'- GGTTACCTTGTTACGACTT-3') based on study by Srinivasan *et al.* (2015) and aerolysin gene primer set: forward primer(5'-CAAGAACAAGTTCAAGTGGCCA-3') and reverse primer(5'-ACGAAGGTGTGGTTCCAGT-3') based on study by Wang *et al.* (2003). The universal primer set was used to amplify bacterial DNA for species identification, while the aerolysin primer set was employed to detect the presence of the aerolysin gene of *Aeromonas hydrophila*.

a) Universal primer sets

PCR was carried out in a Master cycler PCR with the following protocol: Initial denaturation step at 94°C for 1 min, followed by the denaturation phase, where the reaction was heated to 94°C for 40 sec for 35 cycles, then annealing occurred at 56°C for 1 min over 35 cycles, allowing the primers to bind to the target DNA. Afterward, the extension phase took place at 72°C for 1.5 min, repeated for 35 cycles, then a final extension was carried out at 72°C for 7 min.

The PCR products were then sent to First Base Laboratory Sdn. Bhd. for purification and bacterial DNA sequencing. The obtained DNA sequence was edited using BioEdit software to generate the consensus sequence. This consensus sequence was then compared with sequences available in the GenBank database using the BLASTN program to confirm the bacterial species.

b) Aerolysin primer sets

PCR was carried out in a Master cycler PCR with the following protocol: Initial denaturation step at 95°C for 5 min, followed by the denaturation phase, where the reaction was heated to 95°C for 30 sec for 35 cycles, then annealing occurred at 53°C for 30 sec over 35 cycles. Afterward, the extension phase took place at 72°C for 30 sec, repeated for 35 cycles, then a final extension was carried out at 72°C for 7 min.

The reaction results were analysed by electrophoresis using a 1.0% agarose gel to visualize amplification fragments. To stain the gel, 3 µL of Gel Red was added to the agarose solution, and the mixture was poured into the mold. A 1 kb DNA ladder (Biolabs) was used as a molecular size marker, with 5 µL of the ladder loaded into one well. The PCR products, ranging from 2 to 5 µL, were added to the electrophoresis wells. Electrophoresis was performed for 35 min at 90 volts, and the gel was subsequently examined under ultraviolet light using a gel documentation system (Gel Doc).

RESULTS AND DISCUSSION

Analysis of Physicochemical Parameters of the Sea Surface Microlayer

Five replicate water samples and physicochemical measurements were conducted at the same location to ensure data reliability and statistical robustness. The results of the physicochemical parameter analysis of the SML, including total bacterial counts (CFU/mL), at Pulau Tenggol, Terengganu, are presented in Table 1. The temperature range in the study area is between 28.5 and 30.3°C, while the dissolved oxygen (DO) levels range from 7.07 to 7.57 mg/L. The salinity varies between 29.20 and 33.37 ppt, and the pH range is between 8.13 and 8.17. The colony-forming unit (CFU) count revealed the presence of a bacterial population ranging from approximately 0.4×10^4 to 1.4×10^4 CFU/mL in the SML samples.

Table 1. Physicochemical parameters of the sea surface microlayer

Sample	Temperature (°C)	DO (mg/ L)	Salinity(ppt)	pH	Total bacterial count (CFU/ mL)
1	29.1	-	33.37	8.13	1.2×10^4
2	28.5	7.28	30.39	8.15	1.4×10^4
3	29.7	7.57	30.41	8.14	1.2×10^4
4	29.4	7.37	30.01	8.17	0.4×10^4
5	30.3	7.07	29.20	8.16	0.5×10^4

Generally, oxygenated surface microlayer water contains approximately 8 mg/L of dissolved oxygen, a salinity level between 34 and 37 ppt, and a pH range of 7 to 8.5, which is considered optimal for aquatic organisms (Benard *et al*., 2015; Siang *et al*., 2018). In this context, the range of surface water parameters observed in this study closely aligns with those reported by Benard *et al*. (2015) for Mantanani Island, Sabah. Since both islands are significant tourist destinations, they must remain free from contamination, ensuring a safe environment for marine species and preserving their ecological integrity. However, the parameters of the SML are difficult to predict because it has long been recognised as a distinct compartment associated with biogeochemical cycles. This layer represents a highly extreme and dynamic environment, as it is subject to various influences, including fluctuating meteorological conditions, ocean waves, currents, the release of organic compounds from both the atmosphere and the seabed, as well as the presence of diverse microbial life (Skórczewski & Mudryk 2009; Nakajima *et al*., 2013; Engel *et al*., 2017). Besides that, researchers concur that the various characteristics of the SML—such as temperature, pH, dissolved oxygen (DO) levels- are able to influence the diversity of the bacterial population (Aller *et al*., 2005; Lindroos *et al*., 2011; Stolle *et al*., 2011). Furthermore, the influence of the Northeast and Southwest Monsoons is instrumental in shaping the physical and microbiological characteristics of the SML at Tenggol Island, distinguishing it from seawater found at other study locations. (Obernosterer *et al*., 2008; Cunliffe & Murrell 2009; Jaafar *et al*., 2014; Daud & Akhir 2015; Wurl *et al*., 2017).

Table 2. Comparison of pH, temperature, and bacterial presence across different studies

Location	pH	Temperature (°C)	Bacterial count (CFU/mL)	Reference
Pulau Tuba, Langkawi	7.72-8.20	27.43-29.45	0.89×10^3 - 3.4×10^3	Jalal *et al*.,2012
Pulau Mantanani, Sabah	7.23-8.31	27.6-29.8	2.60×10^7	Bernard *et al.,* 2015
Pulau Tenggol	8.31-8.17	28.5-30.3	0.4×10^4 -1.4×10^4	This study

Based on Table 2, the ranges of environmental parameters reported in the studies by Jalal *et al.* (2012) and Benard *et al.* (2015) are comparable to those observed in the present study. Geographically, Pulau Tuba, Pulau Mantanani, and Pulau Tenggol are situated in different regions, with Pulau Tenggol located near the South China Sea and influenced by the Southwest Monsoon during the July sampling period. Several studies have reported an upwelling system along the east coast of Peninsular Malaysia, which brings nutrient-rich waters to the surface and supports high primary productivity (Akhir *et al*., 2015; Chen *et al*., 2020). Therefore, nutrient content should be considered an important factor influencing bacterial abundance in future research. However, despite similar pH and temperature across these sampling sites, there was a notable difference in bacterial abundance. There is an urgent need for further research on the physicochemical properties of the SML and the associated bacterioneuston activity, as current understanding remains limited and often overlooked by the scientific community (Cunliffe *et al*., 2011; Zäncker *et al*., 2018; Wurl *et al*., 2019).

It is also important to note that the physicochemical properties alone are not the sole determinants of bacterioneuston presence. The rapid development of marine tourism on islands has a significant impact on marine water quality, indirectly fostering the growth of pathogenic microorganisms. Research by Hashim *et al*. (2010) highlighted that various tourism activities, such as beach and forest-based recreation, contribute to different forms of pollution. The study also revealed high levels of Oil & Gas (O&G) and Total Suspended Solids (TSS) in the waters of Terengganu, which can be traced to boat traffic, particularly

on Pulau Tenggol, where oil spills and contamination from boats are common. Moreover, the increasing influx of tourists to Pulau Tenggol has led to greater sewage discharge from resorts into the surrounding sea (Zakaria & Hasbullah 2009; Dungun Municipal Council 2019). Both oil spills and sewage discharge are known to increase bacterial diversity, while also promoting the growth of harmful pathogens (Jung *et al*., 2010; Chen *et al*., 2020). As emphasised by Ibrahim *et al*. (2012) and Shukor *et al*. (2017), the implementation of an effective sewage management system is crucial for ensuring the sustainability of tourism while minimising the risk of marine pollution.

Bacterial isolation and analysis

Although total bacterial counts were conducted using nutrient agar with the direct plating method, the preliminary detection of *A. hydrophila* using this medium was not suitable for the intended purpose. This is because nutrient agar is a non-selective medium that supports the growth of a wide range of bacterial species, leading to overcrowded colonies and making the specific isolation of *A. hydrophila* challenging. Moreover, the colony morphology of *A. hydrophila* on nutrient agar closely resembles that of other bacteria, rendering visual identification unreliable. For instance, Kerigano *et al*. (2023) and Patalinghug *et al*. (2022) reported that *A. hydrophila* appears as cream-white colonies on nutrient agar, which was also observed in *Klebsiella pneumoniae*, *Plesiomonas shigelloides,* and was supported by positive control on nutrient agar.

In contrast, modified Rimler-Shotts (mRS) agar, a selective medium, enables more accurate isolation and detection of *A. hydrophila*. Samal *et al*. (2014) and Semwal *et al*. (2023) demonstrated that *A. hydrophila* forms distinct yellow colonies on mRS agar, which was also supported by positive control growth on mRS agar. Numerous studies have supported the effectiveness of mRS agar as a rapid and reliable screening tool for *A. hydrophila*, showing its superiority over other media such as nutrient agar and marine agar in terms of specificity and accuracy (Yogananth *et al*., 2009; Al-Fatlawy & Al-Ammar, 2013; Samal *et al*., 2014). Therefore, in this study, only cream-white single colonies from nutrient agar were randomly selected, while yellow, round, circular colonies observed on mRS agar were specifically isolated for preliminary biochemical testing. This strategy aimed to improve the efficiency and accuracy of identifying *A. hydrophila*.

Colonies cultured on modified Rimler-Shotta (mRS) agar typically exhibit distinct morphological traits, including a round shape, smooth and shiny surface, convex elevation, and yellow coloration. These features strongly indicate the successful isolation of *A. hydrophila*, consistent with the morphology of the positive control (Ahmad *et al*., 2013; Samal *et al*., 2014; Odeyemi & Ahmad 2017; Semwal *et al*., 2023). Figure 1 shows the growth of *A. hydrophila* on mRS agar after streak plating in this study, with clearly visible yellow colonies.

Fig. 1. The growth of *A. hydrophila* on mRS agar.

However, it is important to note that yellow colonies on mRS agar may also indicate the presence of *Vibrio cholerae*, as any bacterium capable of fermenting sucrose can produce a similar coloration (Benedetti & Lippiello, 2003). This resemblance underscores the difficulty of accurate differentiation based solely on colony morphology and highlights the importance of further microscopic and molecular examination to confirm identification. In summary, mRS agar was selected in this study as the primary medium for the targeted isolation of *A. hydrophila*, while nutrient agar was employed for obtaining pure bacterial colonies for subsequent analyses.

Characteristics of *A. hydrophila* through biochemical test

Based on the biochemical tests conducted, the *A. hydrophila* isolates exhibited the following characteristics: Gram-negative (rod-shaped cells), positive for oxidase and catalase, motile, and positive for the indole test, citrate test, methyl red test (M.R.), and Voges-Proskauer test (V.P.). These isolates were also capable of producing carbon dioxide gas and showed resistance to the Vibriostatic disk O/129. However, the *A. hydrophila* isolates did not produce hydrogen sulfide gas. Additionally, one isolate exhibited a red/yellow color (slant/bottom) while the other showed a yellow/yellow color in the TSI test. Nevertheless, both isolates were capable of fermenting glucose. As a result, this study successfully isolated six *Aeromonas* isolates, two of which were preliminarily identified as *A. hydrophila*. Table 3 presents the results of the biochemical tests for the two *A. hydrophila* isolates, known as AH1 and AH2, which correspond to the positive control *A. hydrophila*.

Table 3. Biochemical test results of *A. hydrophila* isolates

	Catalase	Oxidase	Motility	Indol	M.R.	V.P	Citrate	T.S.I.	Glucose	CO_2	H_2S
AH1	-	+	+	+	+	+	+	Y/Y	+	+	-
AH2	-	+	+	+	+	+	+	R/Y	+	+	-
P.C.	-	+	+	+	+	+	+	Y/Y	+	+	-

*T.S.I.: Triple sugar iron; Y: Yellow; R: Red; P.C.: Positive control.

Comparison of the biochemical reactions of *A. hydrophila* isolates from this study with other *A. hydrophila* isolates from previous studies, as shown in Table 4, suggests that different *A. hydrophila* isolates may exhibit distinct biochemical reactions, highlighting the challenges in accurately identifying *Aeromonas* species. Additionally, a review of previous studies reveals that there is no universally agreed-upon set of biochemical tests for identifying *A. hydrophila*. Samal *et al*. (2014) noted that the biochemical test results in their study, as well as those in various other studies, showed minor variations. Aguilera-Arreola *et al*. (2005) also pointed out that *A. hydrophila* exhibits heterogeneous biochemical reactions, and discrepancies often arise between the results of biochemical tests and those from positive bacterial controls (Puthucheary *et al*., 2012). These discrepancies may be due to factors such as the existence of different *A. hydrophila* strains, varying habitat conditions, ecological niches, and potential genetic changes within the bacteria (Stewart *et al*., 2008). Therefore, expanding the range of biochemical tests is essential to improving the accuracy of bacterial identification.

Table 4. Comparison of the biochemical test results from different studies

Test	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	AH1	AH2
Gram stain	-	-	-	-	-	-	-	-	-	-
Catalase	/	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+
Indol	+	/	/	+	/	+	+	+	+	+
H_2S Producer	-	+	+	+	+	-	/	+	-	-
M.R.	/	+	-	+	-	/	-	+	+	+
V.P	+	+	+	+	+	+	+	+	+	+
T.S.I										
a) Colour	Y/Y	R/Y	Y/Y	Y/Y	Y/Y	Y/Y	R/Y	R/Y	Y/Y	R/Y
b) Glucose fermenter	+	+	+	+	+	+	+	+	+	+
CO_2 Producer	/	+	+	/	+	-	/	+	+	+
Vibriostatic disc O/129	+	/	+	+	+	/	/	/	+	+

* (+ = positive, - = negative, / = no data.) (1)= Nahar *et al*., 2016; (2)= Rozi *et al*., 2018; (3)= Rashid *et al*. 2013; (4)= Erdem *et al*., 2011; (5)= Yazdanpanah-Goharrizi *et al*., 2020; (6)= Nielsen *et al*., 2001; (7)= Laith & Najiah 2014; (8)= Thenmozhi *et al*., 2014.

Kusumawaty *et al*. (2016) emphasised the importance of testing for virulence factors, as different strains of *A. hydrophila* may exhibit varying pathogenicity, which can have significant public health implications. In this study, two *A. hydrophila* isolates were shown to produce extracellular enzymes, including proteases, lipases, hemolysins, amylases, and deoxyribonucleases. The virulence factor assays conducted indicated that these isolates could break down proteins, hydrolyze lipids, lyse red blood cells (beta-hemolysin), degrade complex starches, and decompose DNA components. While not all extracellular enzymes contribute to a pathogen's virulence, proteases, lipases, hemolysins, amylases, and deoxyribonucleases have been shown to play a role in disease causation. These extracellular enzymes enhance *A. hydrophila* 's virulence and pathogenicity, facilitating its ability to infect and damage host tissues (Ahmad, 2001; Chenia & Duma, 2017). Figure 2 illustrates the virulence factor tests, such as the amylase test, hemolysis test, and protease test, that were performed in this study. Positive results for all virulence factor tests were based on the observation of clear zones around the isolates. Moreover, studies by Al-Fatlawy and Al-Ammar (2013), Elgendy *et al*. (2017), Al-Fatlawy and Al-Hadrawy (2014), and Samal *et al*. (2014) also utilised biochemical methods to evaluate virulence factors in *A. hydrophila*, with the combined results supporting those observed in the current study.

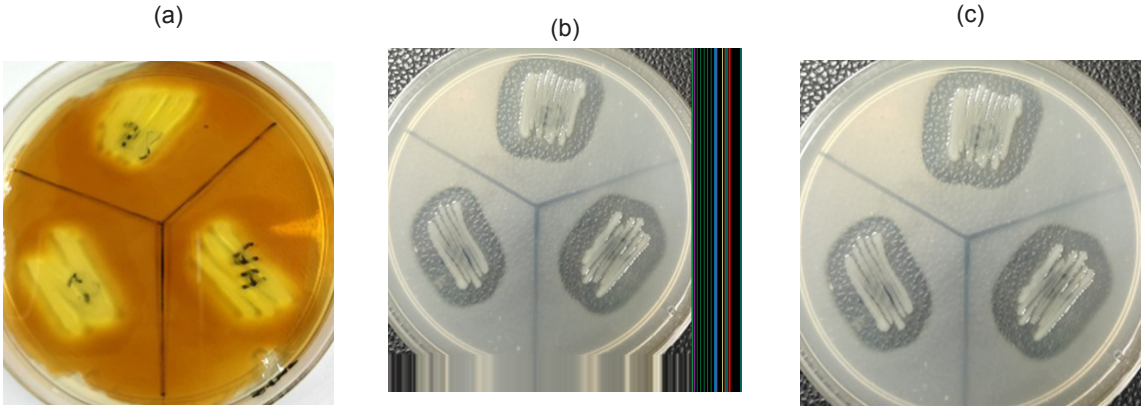

Fig. 2. Virulence factors test. (a)Amylase test; (b) Hemolysis test; (c) Protease test.

In the biofilm test, the microtiter plate machine reading revealed that the absorbance value (OD) for *A. hydrophila* was 0.395 ± 0.0092, while the negative control (ODc) had a value of 0.0523 ± 0.0047. According to the standards (Stepanović *et al*., 2007), *A. hydrophila* in this study demonstrated strong biofilm production ability (OD≥4 ODc). Based on the recorded temperatures shown in Table 2, the SML temperatures (28.5–30.3°C) are conducive to biofilm formation and support the proliferation of *A. hydrophila*. Mizan *et al*. (2018) suggested that temperature can influence biofilm production in *A. hydrophila*, with the optimal temperature for biofilm formation being 20-25°C, achieving an OD of 1.06. However, Mohamed *et al*. (2019) found that *A. hydrophila* produced strong biofilm at 35°C, with an OD of 0.714 ± 0.018. Additionally, Bakhtiari *et al*. (2020), who conducted biofilm assays at 28°C, found that most *A. hydrophila* colonies produced moderate biofilm levels. Interestingly, Chenia and Duma (2017) demonstrated that nutrient concentration plays a more significant role in *Aeromonas* biofilm formation than temperature. Regardless of temperature, as a strong biofilm producer, A. hydrophila benefits from the enhanced protection provided by its biofilm, particularly in the dynamic and fluctuating conditions of the SML. This biofilm not only supports the increased proliferation of *A. hydrophila* but also facilitates its adhesion to a variety of surfaces, such as marine debris or host organisms, trapping essential nutrients to ensure bacterial survival in nutrient-poor environments. Additionally, the biofilm enhances the bacteria's resistance to environmental stresses, antibiotics, and host immune defenses, while also aiding in host cell invasion, which is crucial for its pathogenicity and persistence in aquatic ecosystems.

A. hydrophila strains can be classified into pathogenic and non-pathogenic groups based on their virulence traits (Zhang *et al*., 2000). As noted by Abreu *et al*. (2018), the presence of even a single virulence gene in *A. hydrophila* can render the strain potentially pathogenic. However, Li *et al*. (2011) suggested that the pathogenicity of *A. hydrophila* arises not from a single virulence factor, but from the synergistic interaction of multiple virulence genes. Kidd and Pemberton (2002) demonstrated that amylase activity in *A. hydrophila* is regulated by the coordinated action of several virulence genes. Epple *et al*. (2004) revealed that beta hemolysin is involved in damaging the intestinal epithelium, stimulating active chloride secretion in the intestine, and subsequently causing diarrhea in the host. Furthermore, Ji *et al*. (2015) found that *A. hydrophila* strains lacking the nuclease gene (DNase) are more susceptible to destruction by fish macrophages, indicating the importance of this gene in immune evasion. Meanwhile, Hamid *et al*. (2016) demonstrated that hemolytic strains of *A. hydrophila* can induce fish mortality.

Antibiotic susceptibility

In the present study, antibiotic resistance testing was carried out to evaluate the response of *A. hydrophila* isolates obtained from the SML to nine different antibiotics. The two *A. hydrophila* isolates in this study were susceptible only to chloramphenicol (30 µg), while they exhibited resistance to colistin sulfate (10 µg), ampicillin (10 µg), oxytetracycline (30 µg), penicillin (110 µg), polymyxin B (300 µg), erythromycin (15 µg), nalidixic acid (30 µg), and sulfamethoxazole (25 µg). The inhibition zone diameters for the two *A. hydrophila* isolates against chloramphenicol were 28 mm and 22 mm, indicating susceptibility, as a zone diameter exceeding 18 mm denotes sensitivity to chloramphenicol. The MAR index recorded was >0.2, suggesting that the isolates originated from a source that has antibiotic contamination.

The *A. hydrophila* isolates in this study were susceptible only to chloramphenicol, a broad-spectrum antibiotic frequently used in Malaysian fish farms. Similar findings were reported by Park *et al*. (2011) and Ali *et al*. (2016). Despite its efficacy, chloramphenicol was banned by the USFDA in 1984 due to its potential to cause aplastic anemia when residues remain in food products. This led to the rejection of shrimp shipments from Malaysia in 2014-2015 due to chloramphenicol contamination (Loh *et al*., 2020). In response, Malaysia enacted the Aquaculture Fisheries (Land) Regulations 2017, prohibiting the use of antibiotics and pesticides in aquaculture, while the Ministry of Health and the Ministry of Agriculture and Agro-based Industry launched the Malaysian Antimicrobial Resistance Action Plan to regulate antibiotic use across industries (Ministry of Health Malaysia, 2017). The Environmental Protection Agency (EPA) has included *A. hydrophila* on the Contaminant Candidate List, thereby prompting routine monitoring of its presence in water and food supplies (Borchardt *et al*., 2003). Despite these challenges, antibiotics remain a primary treatment option for *A. hydrophila* infections.

A. hydrophila has long been recognized for its remarkable resistance to a wide array of antibiotics. Strains exhibiting high resistance to multiple antibiotics often demonstrate increased pathogenicity (Galindo *et al*., 2006). The mechanisms of virulence and antibiotic resistance are closely linked, as both serve to enhance pathogenicity and bacterial survival. The study suggests that the emergence of multidrug-resistant *A. hydrophila* strains may result from horizontal gene transfer of antibiotic resistance genes from other highly resistant pathogens, mediated by plasmids, transposons, and integrons, rather than solely from antibiotic contamination (Evangelista-Barreto *et al*., 2010; Beceiro *et al*., 2013; Stratev & Odeyemi, 2016). Magiorakos *et al*. (2012) classify

bacteria resistant to at least one antibiotic class as multidrug-resistant strains. According to the World Health Organization (2019), polymyxins, penicillins, tetracyclines, macrolides, fluoroquinolones, and sulfonamides are six distinct antibiotic classes. In this study, *A. hydrophila* exhibited resistance to multiple antibiotic classes, confirming its classification as a multidrug-resistant strain. Evangelista-Barreto *et al*. (2010) and Sreedharan *et al*. (2012) emphasised that *A. hydrophila* strains resistant to multiple antibiotics should be managed with caution to prevent environmental spread and reduce the risk of consuming contaminated food and beverages. The challenge of treating infections caused by these resistant strains is exacerbated by the ineffectiveness of commonly used antibiotics in inhibiting bacterial proliferation, leading to significant public health concerns. The presence of multidrug-resistant *A. hydrophila* in the SML poses a risk of contamination to food sources and can cause illness in individuals who come into contact with polluted seawater, thus threatening public health and safety.

Identification of *A. hydrophila* through PCR

In this study, PCR reactions were performed using the 16S rRNA universal primer set and also the aerolysin (aerA) primer set on the six *Aeromonas* isolates. As a result, the universal primer successfully confirmed that isolates AH1 and AH2 were *Aeromonas hydrophila*, showing a 99% sequence identity match with *A. hydrophila* reference sequences available in the GenBank database (Gene sequences of AH1 & AH2 were provided in the Appendix section). Notably, species such as *Staphylococcus aureus*, *Escherichia coli*, *Vibrio alginolyticus*, *Aeromonas caviae*, and *Aeromonas taiwanensis* do not produce aerolysin, and thus, PCR amplification targeting this gene would not yield products for these organisms. Based on Figure 3, the presence of a DNA band for the aerA gene of *A. hydrophila* isolates appears at 309 bp, as revealed under UV light following the electrophoresis process.

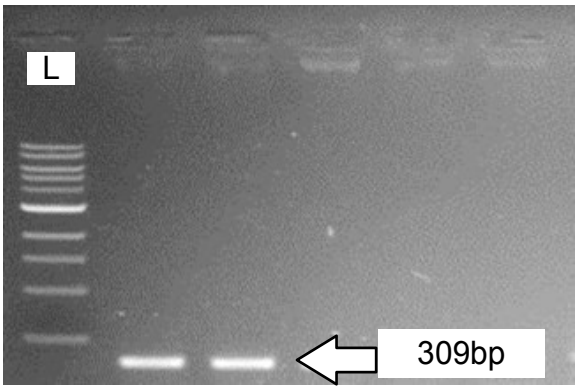

Fig. 3. Electrophoresis gel showed *A. hydrophila* isolates that contain the aerA gene. (L Ladder 1kb)

Many researchers from various fields, such as food control and safety, zoology, and human health, have also used the aerA primer to detect the presence of *A. hydrophila* and test the ability of *A. hydrophila* to produce aerolysin (Wejdan *et al*., 2014; Furmanek-Blaszk, 2014; Oleiwi *et al*., 2014; Stratev *et al*. 2016; Hamid *et al*., 2016; Zhou *et al*., 2018). Radosavljević *et al*. (2013) and Christy *et al*. (2019) affirmed that the aerA gene functions as a key genetic marker for evaluating *A. hydrophila*'s virulence and pathogenic potential. According to El Deen *et al*. (2014), aerolysin serves as a critical virulence factor due to its potent proteolytic activity, which can be lethal to the host. Aerolysin's capacity to independently initiate disease and contribute to mortality, particularly in poikilothermic animals, has attracted substantial attention within the scientific community (Abrami *et al*., 2000; Singh *et al*., 2010; Zhang *et al*., 2013). The presence of aerolysin is an essential determinant of pathogenicity in *A. hydrophila*, marking it as a major virulence factor. Multiple studies have demonstrated the detrimental effects of aerolysin on animal models, showing its ability to damage epithelial and red blood cells upon expression of the *A. hydrophila* aerolysin gene in *E. coli* (Epple *et al*., 2004; Singh *et al*., 2008; Bücker *et al*., 2011; Zhang *et al*., 2013). The ability of *A. hydrophila* to produce aerolysin poses significant challenges to the aquaculture industry, as pathogenic strains are known to cause rapid mortality in fish. This issue is of particular concern in Malaysia, where the fisheries sector is expanding rapidly and making substantial contributions to the national economy.

Comparison of findings between biochemical tests and PCR

The findings highlight that while culture media alone can lead to inaccurate or misleading results, the combination of traditional microbiological methods and PCR provides a more precise and reliable means of identifying *A. hydrophila*. In this study, both biochemical tests and PCR reactions were utilised for the identification of *A. hydrophila*, a method widely adopted in previous research to detect this bacterium (Yogananth *et al*., 2009; Odeyemi *et al*., 2012; Al-Fatlawy & Al-Hadrawy, 2014; Tran *et al*., 2015; Othman *et al*., 2017). In this study, culture-based virulence factor assays demonstrated the virulence potential of *A. hydrophila*, while PCR analysis further confirmed the presence of the aerolysin (aerA) gene in the isolates. Together, these findings support the potential pathogenicity of the identified *A. hydrophila* strains.

Although biochemical tests have long been used for bacterial identification, their limitations in accurately identifying closely related strains have prompted many researchers to favor PCR-based methods. For example, in the study by Figueras Salvat and Ashbolt (2019), biochemical tests led to the misidentification of *Aeromonas dhakensis* as either *A. hydrophila* or *Aeromonas aquariorum*. *A. hydrophila* may exhibit varying colony colors on nutrient agar (Pham *et al*., 2003; Thenmozhi *et al*., 2014, Tran *et al*., 2015) and tryptic soy agar (Nielsen *et al*., 2001; Nahar *et al.,* 2016) in different studies, leading to uncertainty regarding the reliability of culture media for accurate bacterial identification. The emergence of new *Aeromonas* strains and

the similarity in biochemical profiles between closely related bacterial species have added complexity to the identification process (Tomás, 2012; Mzula *et al*., 2019a). Additionally, factors such as low bacterial concentration, unrepresentative sampling, the presence of bacteria in a viable but non-culturable state (or dead), contamination during culture, and human error (e.g., inaccurate observations) may impede the isolation of *A. hydrophila* from seawater samples, thereby compromising the precision and accuracy of the culture method.

PCR is highly effective for detecting *A. hydrophila* in the SML, providing more accurate results than traditional biochemical tests. Its speed and precision make it the preferred method for monitoring *A. hydrophila*, offering clear advantages in efficiency and accuracy. This method not only enables quicker and more reliable diagnoses but also reduces laboratory processing time and streamlines the screening process. As noted by Erdem *et al*. (2011), PCR consistently demonstrates higher accuracy and precision compared to biochemical tests. Furthermore, PCR's ability to detect both viable *A. hydrophila* cells and non-culturable or dead bacteria makes it a more comprehensive and reliable diagnostic tool. This is especially critical given *A. hydrophila*'s role in causing diseases in fish farms and its association with diarrheal illnesses in children in Malaysia (Lee & Puthucheary, 2002; Laith & Najiah, 2014). Consequently, PCR testing facilitates timely, targeted treatments for both humans and animals, playing a pivotal role in reducing mortality rates and preventing the spread of disease outbreaks. With the rapid advancements in modern technology, various innovative methods, such as Real-Time PCR and Loop-Mediated Isothermal Amplification (LAMP), have been developed to facilitate on-site testing and provide immediate results. These techniques offer promising potential for detecting *A. hydrophila* and other pathogens in the SML, where the unique environmental conditions contribute to the distinctive properties of bacterianeuston.

CONCLUSION

This study successfully isolated two potentially pathogenic *A. hydrophila* strains from the SML at Pulau Tenggol, Terengganu. Both isolates were characterised and identified, with results indicating that they produce extracellular enzymes (proteases, lipases, hemolysins, amylases, and deoxyribonucleases), are strong biofilm formers, and exhibit multiple antibiotic resistances. These characteristics highlight the ability of *A. hydrophila* to persist in marine environments and potentially adapt to changing environmental conditions, including anthropogenic influences. Given Pulau Tenggol's popularity for water-based recreational activities such as scuba diving, the detection of such resilient and potentially harmful bacteria underscores the importance of proactive environmental monitoring. Continuous assessment of the SML will not only support early pathogen detection and safeguard public health but also contribute to understanding microbial dynamics in this ecologically sensitive interface. Furthermore, the findings of this study establish a valuable baseline for tracking changes in microbial composition over time, which could serve as an indicator of shifts in water quality or the impact of climate-related and human-induced stressors. As studies specifically focusing on *A. hydrophila* in the SML remain scarce, future research should aim to integrate molecular, metagenomic, and environmental data to provide a more comprehensive understanding of this unique habitat and its public health implications.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to University Kebangsaan Malaysia and the financial support from the Grant.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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