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Antibiotic Resistance and Virulence Gene Profiles of *Vibrio parahaemolyticus*, *Vibrio cholerae*, and *Vibrio alginolyticus* Isolated from Commercial Shrimp Farm in Kuching, Sarawak

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ABSTRACT

In the management and treatment of *Vibrio* spp. infections in aquaculture, antibiotics have traditionally been used. Misuse of antibiotics, however, has led to the emergence of resistance strains. In this study, antibiotic susceptibility testing of 30 (n=30) *Vibrio* spp. isolates were performed by using 18 antibiotics, revealing resistance to at least two antibiotics. Antibiotics Ceftazidime, Meropenem, Gentamicin, Tetracycline, Nalidixic acid, Norfloxacin, Ciprofloxacin, and Chloramphenicol were 100% effective against all isolates of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus*. Meanwhile, 100% of *V. parahaemolyticus* and *V. alginolyticus* isolates were completely resistant to Penicillin G and Bacitracin, whereas 100% of *V. cholerae* isolates exhibited resistance to Penicillin G. The Multiple Antibiotic Resistance (MAR) indices of all isolates ranged from 0.11 to 0.33. The presence of isolates with MAR indices higher than 0.2 suggests potential contamination from sources with high antibiotic usage, such as wastewater or nearby agricultural and aquaculture activities. The findings highlight widespread antibiotic resistance among *Vibrio* spp., likely due to excessive antibiotics use in aquaculture settings. Additionally, virulence profile of each *Vibrio* spp. isolates was performed. While pathogenic potential is exhibited by some isolates, others lack key virulence genes associated with pathogenicity. All *V. parahaemolyticus* isolates showed the presence of *tlh*, *toxR*, and *toxS* genes, whille all *V. cholerae* isolates were positive with *toxS*, *toxR*, *rtxA*, and *rtxC* genes. None of the *V. alginolyticus* showed the presence of the nine tested virulence genes. However, given the high frequency of horizontal gene transfer among bacterial populations, continuous and comprehensive monitoring is crucial to prevent the spread of virulence genes between pathogenic and non-pathogenic strains. Therefore, continuous efforts to obtain more data on antibiotic resistance and bacterial virulence profiles in Sarawak is crucial for effective disease management and sustainable aquaculture practices.

Keywords: Antibiotic resistance, shrimp, virulence genes, *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*

INTRODUCTION

Controlling the population of *Vibrio* spp. presents significant challenges attributable to their pathogenicity and antibiotic resistance. These bacteria can cause diseases in both marine organisms and humans (Sampaio *et al*., 2022). Furthermore, the emergence of antibiotic-resistant strains of *Vibrio* spp. is increasingly worrisome, given the diminished efficacy of conventional treatments employing broad-spectrum antibiotics. Moreover, *Vibrio* spp. is acknowledged for possessing the highest growth rate among bacteria, and their adeptness at thriving in various environmental conditions, contributes to their resilience and persistence (Baker-Austin *et al*., 2020; Abioye *et al*., 2021).

In aquaculture, farmers commonly use antibiotics to treat bacterial infection, particularly vibriosis caused by *Vibrio* species. In shrimp farming, antibiotics such as oxytetracycline, tetracycline, quinolones, sulfonamides, enrofloxacin, norfloxacin, gentamicin, and trimethoprim are routinely used to reduce shrimp morbidity and mortality (Haifa-Haryani *et al*., 2022). In Malaysia, antibiotics are administered either via inclusion in feed additives or through immersion baths, serving as both prophylactic measures and therapeutic interventions for infected organisms (Ibrahim *et*

al., 2010; Amalina *et al*., 2019). However, excessive antibiotic use has led to reduced efficacy and the emergence of antimicrobial-resistant (AMR) strains. Furthermore, the spread of AMR bacteria may be facilitated across different geographic regions through the import and export of live aquatic animals and animal-derived products (Rodgers *et al*., 2011; Paria *et al*., 2021).

Beyond antibiotic resistance, identifying and monitoring virulence genes in *Vibrio* spp. helps assess their pathogenicity and improve disease management. *Vibrio* spp. infections have been documented as major contributors to instances of foodborne illness in Malaysia. *Vibrio parahaemolyticus*, in particular, is reported to be the cause of the highest seafood-associated gastroenteritis in various Asian countries (Elmahdi *et al*., 2016). Consumption of *Vibrio*-contaminated food products can result in gastroenteritis and may even lead to septicaemia and mortality in immunocompromised individuals (Abdullah Sani *et al*., 2013; Song *et al*., 2020). Additionally, the global occurrence of *V. parahaemolyticus* prompts efforts to understand their pathogenicity and their interactions with humans and aquatic animals (Wang *et al*., 2015). Although the majority of environmental isolates of *Vibrio* spp. are non-pathogenic, their genomes are highly dynamic, meaning that non-pathogenic strains can easily acquire virulence genes from pathogenic strains through horizontal gene transfer (Xu *et al*., 2017). Consequently, *Vibrio* spp. maintain an extensive reservoir of virulence genes, even in non-pathogenic strains (Zoqratt *et al*., 2018; Abdelaziz Gobarah *et al*., 2022).

Antibiotic resistance poses a substantial challenge to public health and economic stability. These issues underscore the urgent need for effective strategies to combat antibiotic resistance, as it is a growing threat with far-reaching implications. Efforts to mitigate these challenges require a multifaceted approach, including an improved understanding of *Vibrio* spp. biology, enhanced surveillance and monitoring, and the development of alternative treatment modalities to combat infections caused by these bacteria. The emergence of resistant strains not only compromises the efficacy of antibiotic treatment but also raises concerns about the potential for increased morbidity, mortality, and healthcare risks and costs (WHO, 2023). Moreover, antibiotic resistance transcends national borders, necessitating collaborative efforts across countries and sectors to address this pressing issue (Muteeb *et al*., 2023). As recognized by the WHO, antibiotic resistance poses a significant threat to global health security, emphasizing the critical importance of proactive measures to mitigate its impact (WHO, 2023).

Therefore, it is imperative to monitor and control the dissemination of AMR strains within a farm to limit their spread and aid in disease management. Consequently, this study expands on our previous findings on the prevalence of *Vibrio* spp. in a commercial shrimp farm (Lesen *et al*., 2024) by further examining the antibiotic resistance and virulence gene profiles of three *Vibrio* spp. isolated from the Telaga Air shrimp farm.

MATERIALS AND METHODS

Isolation and Enrichment of Bacteria

Samples were collected from Persatuan Nelayan Kawasan (PNK) Satang Biru, Lembaga Kemajuan Ikan Malaysia (LKIM), Telaga Air, Sarawak (0''N 110°11'51., 1°40'59, 1). Four sample types (water, sediment, shrimp, and effluent) were collected from two shrimp ponds. Overall, a total of 48 (n = 48) samples were collected from the shrimp farm over a single production cycle and the prevalence of *Vibrio* spp. were studied (Lesen *et al*., 2024). The sampling method was derived from the methodology outlined by Kaysner *et al*. (1990) with adaptations. During each sampling event, each sample type was collected from three distinct locations (sampling points) to constitute triplicates. Surface water and effluent samples, each amounting to at least 1 L, were collected in sterile polypropylene bottles at each sampling point. Shrimp were captured using a net and transferred to sterile plastic bags. Meanwhile, sediment was gathered using an ethanol-sterilized polyvinyl chloride (PVC) pipe and stored in sterile 50 mL microcentrifuge tubes. All samples were promptly transported to the laboratory in an ice box and processed within 24 hours of collection (Kaysner *et al*., 1990).

Before enrichment, the sediment and shrimp samples were homogenised using a sterilised conventional blender. One gram (for solid

samples) or 1 mL (for liquid samples) of each sample was thoroughly mixed with 9 mL of alkaline peptone water (APW) (Merck, Darmstadt, Germany) and pre-enriched through overnight incubation at 37 °C. Subsequently, the enriched cultures were spread and streaked on differential and selective media, Thiosulfate–citrate–bile salts–sucrose (TCBS) agar (Himedia, Mumbai, India) and CHROMAgar™ *Vibrio* (CHROMagar™, Paris, France), to acquire single colonies. On TCBS agar, *Vibrio parahaemolyticus* would appear as green colonies, while *V. cholerae* and *V. alginolyticus* would appear as yellow colonies. Meanwhile, on CHROMAgar™ *Vibrio*, *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* appear as mauve, blue, and milky white colonies, respectively.

Based on these morphologies, 10 pure isolates (n=10) of each *Vibrio* spp. were randomly selected and cultured in APW. The presumptive *V. parahaemolyticus* isolates were assigned codes TA01 – TA10, *V. cholerae* isolates as TA11 – TA20, and *V. alginolyticus* isolates as TA21 – TA30 (The source of each isolate is reported in Table 4, 5, and 6 in the Results section). Multiple rounds of culturing and streaking on selective media were conducted to ensure the isolation of pure cultures.

Confirmation of Isolates using Species-Specific Multiplex PCR

PCR was performed using species-specific primer sets to confirm the identity of each isolate. The DNA of each isolate was extracted by using cell-boiled method (Queipo-Ortuno, 2008). Multiplex PCR was conducted using three sets of primers that specifically targeted *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus*, as designed by Kim *et al*. (2015). The primers used are shown in Table 1 below. Each 15 µL of the PCR mixture consisted of the following components: 7.5 µL exTEN 2X PCR Master Mix (1st BASE, Singapore), 0.6 µL of each primer with a concentration of 10 µM, 2.0 µL DNA template, and 1.9 µL sterile distilled water.

PCR amplification was performed using the T100™ Thermal Cycler (Bio-Rad, USA) following the specified conditions: initial denaturation at 94 °C for 5 minutes; 30 cycles comprising denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds; and a final extension step at 72 °C for 10 minutes (Kim *et al*., 2015). Subsequently, the PCR products were subjected to electrophoresis on a 1.5% (w/v) agarose gel at 80 V for one hour. The GeneRuler 100-bp and 1-kbp DNA ladders (Thermo Fisher Scientific, USA) were used as the molecular weight marker. A mixture of *V. parahaemolyticus* ATCC 27969, *V. cholerae* KCDC 13589, and *V. alginolyticus* ATCC 17749 served as the positive control, while sterile APW processed concurrently with the samples was utilised as the negative control to substitute the DNA template.

Antibiotic Susceptibility Test (AST)

Once the identity of each isolate was confirmed, the susceptibility of each isolate to different antibiotics was assessed using the disc diffusion assay, as outlined by the Clinical and Laboratory Standards Institute (Hudzicki, 2009; Clinical and Laboratory Standard Institute, 2015). The bacterial isolates were cultured in 10 mL Mueller-Hinton Broth (MHB) supplemented with 2% NaCl at 37 °C for 24 hours.

The following day, the overnight cultures were adjusted to a 0.5 McFarland standard. Using sterile cotton swabs, the cultures were spread onto Mueller-Hinton agar (MHA) plates supplemented with 2% NaCl and allowed to dry for five minutes. Then antibiotic discs were placed on the agar surface in triplicate using sterile forceps. The discs were positioned at approximately equal distances from each other to ensure accuracy. The antibiotics used in this susceptibility test included Ampicillin (AMP, 10 µg), Chloramphenicol (C, 30 µg), Penicillin G (P, 10 IU), Amoxicillin-clavulanic acid (AMC, 30 µg), Amikacin (AK, 30 µg), Erythromycin (E, 15 µg), Tetracycline (TE, 30 µg), Ceftazidime (CAZ, 30 µg), Ciprofloxacin (CIP, 5 µg), Cephalotin (KF, 30 µg), Norfloxacin (NOR, 10 µg), Gentamicin (CN, 10 µg), Rifampicin (RD, 5 µg), Imipenem (IPM, 10 µg), Kanamycin (K, 30 µg), Nalidixic acid (NA, 30 µg), Bacitracin (B, 10 IU), and Meropenem (MEM, 10 µg) (Oxoid, Hampshire, United Kingdom). These antibiotics are commonly employed to treat *Vibrio* spp. infections (Elmahdi *et al*., 2016). *Escherichia coli* ATCC® 25922 was used as control. All plates were then incubated at 37 °C for 24 hours.

Following incubation, the diameter of the antibiotic inhibition zone was measured, and the average was calculated based on triplicate measurements. The level of susceptibility was determined according to the guidelines in the Clinical and Laboratory Standards Institute's Document M-45 for *Vibrio* species (Clinical and Laboratory Standard Institute, 2015). From the susceptibility results, the Multiple Antibiotic Resistant (MAR) indices of the isolates were determined using the following formula Eq.(1):

$$\text{MAR index} = \frac{\text{Number of antibiotics the isolate}}{\text{Total number of antibiotic}} \quad \text{Eq. (1)}$$

Polymerase Chain Reactions (PCR) for Virulence Genes Detection

The DNA of each bacterial isolate was extracted using the boiled-cell method (Queipo-Ortuno, 2008). Multiplex and singleplex PCRs were conducted using nine primer sets targeting different virulence genes of *Vibrio* spp., as outlined in Table 2.

Table 1. Sequences, sources, and expected amplicon sizes of primer pairs used to target each *Vibrio* spp. in this study (Kim *et al*., 2015)

Target species	Primer name	Primer sequence (5'→ 3')	Product size (bp)
Vibrio parahaemolyticus	VP 1155272 F	AGCTT ATTGG CGGTT TCTGT CGG	297
	VP 1155272 R	CKCAA GACCA AGAAA AGCCG TC	
Vibrio cholerae	VC C634002 F	CAAGC TCCGC ATGTC CAGAA GC	154
	VC C634002 R	GGGGC GTGAC GCGAA TGATT	
Vibrio alginolyticus	VA 1198239 F	ACGGC ATTGG AAATT GCGAC TG	199
	VA 1198239 R	TACCC GTCTC ACGAG CCCAA G	

Table 2. List of primers targeting different virulence genes used in this study

Virulence gene	Primer name	Primer sequence (5'→ 3')	Product size (bp)	Annealing temperature (°C)	Reference
tdh	F-*tdh*	GTA AAG GTC TCT GAC TTT TGG AC	269	58	Bej *et al*. (1999)
	R-*tdh*	TGG AAT AGA ACC TTC ATC TTC ACC			
trh	F-*trh*	TTG GCT TCG ATA TTT TCA GTA TCT	500	58	Bej *et al*. (1999)
	R-*trh*	CAT AAC AAA CAT ATG CCC ATT TCC G			
tlh	tl-F	AAA GCG GAT TAT GCA GAA GCA CTG	450	58	Bej *et al*. (1999)
	tl-R	GCT ACT TTC TAG CAT TTT CTC TGC			
pirA	AP3-F	ATG AGT AAC AAT ATA AAA CAT GAA AC	333	53	Sirikharin *et al*. (2015)
	AP3-R	GTG GTA ATA GAT TGT ACA GAA			
V. cholerae-associated *toxS*	F-*toxS*	CCA CTG GCG GAC AAA ATA ACC	640	52	Sechi *et al*. (2004)
	R-*toxS*	AAC AGT ACC GTA GAA CCG TGA			
V. cholerae-associated *toxR*	F-*toxR*	TTT GTT TGG CGT GAG CAA GGT TTT	595	52	Sechi *et al*. (2004)
	R-*toxR*	GGT TAT TTT GTC CGC CAG TGG			
rtxA	*rtxA*-F	CTG AAT ATG AGT GGG TGA CTT ACG	417	55	Chow *et al*. (2001)
	rtxA-R	GTG TAT TGT TCG ATA TCC GCT ACG			
rtxC	*rtxC*-F	CGA CGA AGA TCA TTG ACG AC	263	55	Chow *et al*. (2001)
	rtxC-R	CAT CGT CGT TAT GTG GTT GC			
ctxB	ctx B_2	GAT ACA CAT AAT AGA ATT AAG GAT G	460	55	Chow *et al*. (2001)
	ctx B_3	GGT TGC TTC TCA TCA TGG AAC CAC			

The detection of *tdh*, *trh*, and *tlh* genes was carried out using a multiplex PCR method adapted from Bej *et al*. (1999), with minor adjustments. Each PCR reaction mixture (25 µL) comprised 12.5 µL of exTEN 2X PCR Master Mix (1st BASE), 1.25 µL of each 10 µM primer, 2.0 µL of DNA extract, and 3.0 µL of sterile distilled water. PCR conditions included initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10

min (Bej *et al*., 1999). A clinical isolate of *V. parahaemolyticus* (from laboratory stock), previously confirmed to possess the *tlh*, *tdh*, and *trh* genes, was used as the positive control.

The detection of the *pirA* gene was performed using a singleplex PCR under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C for 30 sec, extension at 72 °C for 40 sec, and a final extension at 72 °C for 7 min. The PCR mixture consisted of 7.5 µL of exTEN 2X PCR Master Mix (1st BASE, Singapore), 1.0 µL of each forward and reverse AP3 primer (10 µM concentration), 2.0 µL of DNA extract, and 5.5 µL of distilled water (Sirikharin *et al*., 2015). An environmental isolate of *V. parahaemolyticus* (from laboratory stock), previously confirmed to possess the *pirA* genes, was used as the positive control.

Meanwhile for the detection of *V. cholerae*-associated *toxR* and *toxS* genes, a multiplex PCR was performed following the method described by Sechi *et al*. (2004). The PCR mixture included 12.5 µL of exTEN 2X PCR Master Mix (1st BASE, Singapore), 1.0 µL of each 10 µM primer, 2.0 µL of DNA template, and 4.5 µL of sterile distilled water. PCR conditions were similar to those used for detecting *tdh*, *trh*, and *tlh* genes, with the annealing temperature adjusted to 52 °C and the final extension extended to 20 min (Sechi *et al*., 2004). A clinical isolate of *V. cholerae* that has been confirmed for the presence of *toxR* and *toxS* genes was used as the positive control.

Additionally, detection of *rtxA*, *rtxC*, and *ctxB* genes was conducted using a multiplex PCR mixture containing 12.5 µL of exTEN 2X PCR Master Mix (1st BASE, Singapore), 0.6 µl of each primer (10 µM concentration), 2.0 µL of DNA extract, and 6.9 µL of distilled water. The PCR protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min (Chow *et al*., 2001). A clinical isolate of *V. cholerae* that was confirmed for the presence of *rtxA*, *rtxC*, and *ctxB* genes was used as the positive control.

In all PCRs mentioned above, sterile distilled water (dH_2O) was used as the negative control. The reactions were conducted using the T100™ Thermal Cycler (Bio-Rad, USA). PCR products were visualised through agarose gel electrophoresis (AGE) with a 1.5% gel concentration. Electrophoresis was carried out at 80 V for 2 hours, and the gels were then stained with Ethidium bromide (EtBr) for one hour before visualisation using a safeVIEW-MINI2 Blue Light Transilluminator (Cleaver Scientific, Warwickshire, United Kingdom). The presence of bands was observed and recorded.

RESULTS

Isolation of *Vibrio* species

When plated on selective and differential media, such as TCBS and CHROMAgar™ *Vibrio*, different *Vibrio* spp. bacterial colonies appear with different coloured morphologies (Figure 1). *Vibrio parahaemolyticus* appeared as green colonies on TCBS agar, while *V. cholerae* and *V. alginolyticus* both appeared as yellow colonies (Figure 1a). Meanwhile, *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* appeared as mauve, blue, and milky white colonies, respectively, on CHROMAgar™ *Vibrio* (Figure 1b).

The anticipated sizes for the PCR products of *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholerae using* the primer sets outlined in Table 1 are 297 bp, 199 bp, and 154 bp, respectively. Agarose gel electrophoresis (AGE) of the 30 isolates revealed bands that were consistent with the expected sizes. Based on PCR, it has been confirmed that each of the selected isolates were indeed their respective *Vibrio* species (Figure 2).

Antibiotic Susceptibility Test (AST) of *Vibrio* species

The antibiotic susceptibility test was performed using 18 antibiotics that are commonly used for controlling *Vibrio* spp. infections (Elmahdi *et al*., 2016). The responses of the isolates were categorised as susceptible (can be treated with the antibiotic), intermediate (may be treated with adjusted dosage), and resistant (cannot be treated with the antibiotic) based on guidelines from the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standard Institute, 2015; CDC, 2019a). The test revealed varying responses of each isolate to the tested antibiotics.

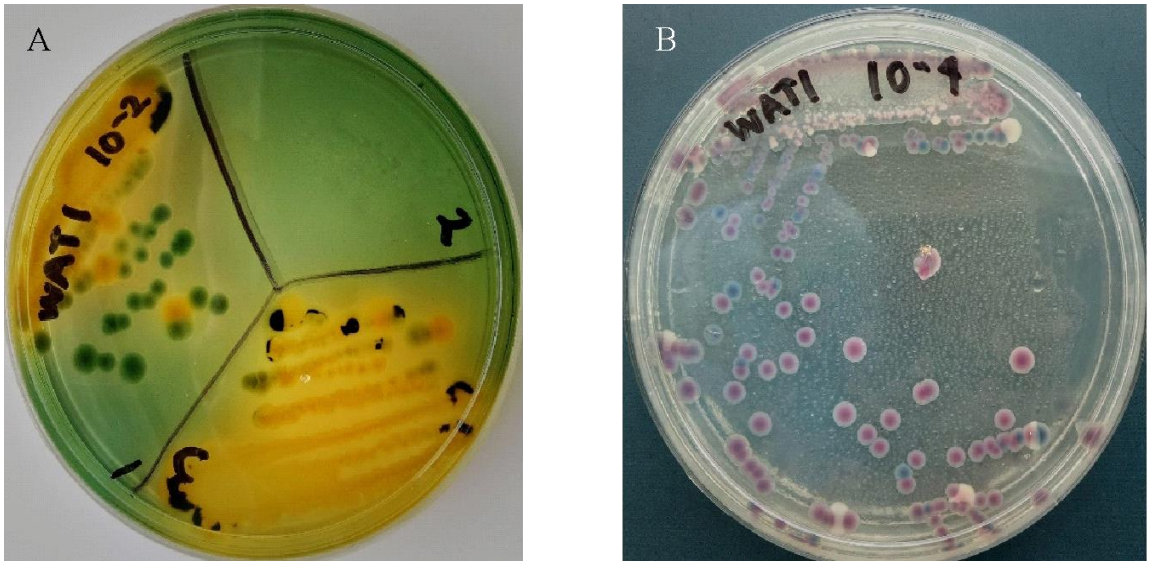

Figure 1. *Vibrio* spp. appearances on TCBS and CHROMAgar™ *Vibrio*. (a) *Vibrio parahaemolyticus* appears as green colonies, while V. cholerea and *V. alginolyticus* appear as yellow colonies on TCBS agar. (b) *Vibrio parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* appear as mauve (purple), blue, and white colonies, respectively on CHROMAgar™ *Vibrio*

Analysis of Table 3 below indicates that Ceftazidime, Meropenem, Gentamicin, Tetracycline, Nalidixic acid, Norfloxacin, Ciprofloxacin, and Chloramphenicol were 100% effective against all isolates of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus*. Amoxicillin-clavulanate was effective against 100% of *V. parahaemolyticus* and *V. cholerae* isolates, while Erythromycin was 100% effective against all isolates of *V. parahaemolyticus* and *V. alginolyticus*. Conversely, it was observed that 100% of *V. parahaemolyticus* and *V. alginolyticus* isolates were completely resistant to Penicillin G and Bacitracin, whereas 100% of *V. cholerae* isolates exhibited resistance to Penicillin G.

Based on observations made during the measurement of the zone of inhibition after 24 hours of incubation, it was noted that a few isolates exhibited colony growth within the existing zone of inhibition, as depicted in Figure 3 below. Following several repeated tests using freshly subcultured pure isolates, consistent results were obtained. Therefore, these isolates were classified as resistant to the antibiotics, irrespective of the diameter of the inhibition zone. This growth within the zone of inhibition indicated the presence of resistant mutants (Hudzicki, 2009).

Multiple Antibiotic Resistance (MAR) Profiles

The antibiotic resistance profiles and MAR indices of *V. parahaemolyticus* isolates from the shrimp farm are detailed in Table 4 below. Eight distinctive antibiotic resistance patterns were observed among the *V. parahaemolyticus* isolates, with 2 isolates (*V. parahaemolyticus* TA01 and TA10) exhibiting the same resistance pattern of P-B, and another 2 isolates (*V. parahaemolyticus* TA02 and TA03) displaying an antibiotic resistance pattern of P-AK-KF-B. The remaining isolates exhibited varying patterns. Isolate TA01 and TA10 were respectively isolated from water and effluent samples, while isolate TA02 and TA03 were both isolated from water samples. The MAR indices of the *V. parahaemolyticus* isolates ranged from 0.11 to 0.39. Pattern P-B demonstrated the lowest MAR index (0.11), whereas pattern AMP-P-AK-KF-RD-IPM-B, belonging to isolate TA07 previously isolated from a shrimp sample, exhibited the highest MAR index (0.39).

In Table 5 below, the antibiotic resistance profiles and MAR (Multiple Antibiotic Resistance) indices of ten *V. cholerae* isolates from the shrimp farm are presented. Eight

distinctive antibiotic resistance patterns were observed, with 2 isolates (*V. cholerae* TA11 and TA12) exhibiting the same resistance pattern of P-KF-RD-IPM-B, while another 2 isolates (*V. cholerae* TA16 and TA18) displayed a resistance pattern of P-RD-B. The remaining isolates showed varying patterns from each other. The MAR indices of the *V. cholerae* isolates ranged from 0.11 to 0.33. Similar to the *V. parahaemolyticus* isolates, pattern P-B exhibited the lowest MAR index (0.11), while pattern AMP-P-E-KF-RD-B demonstrated the highest MAR index (0.33).

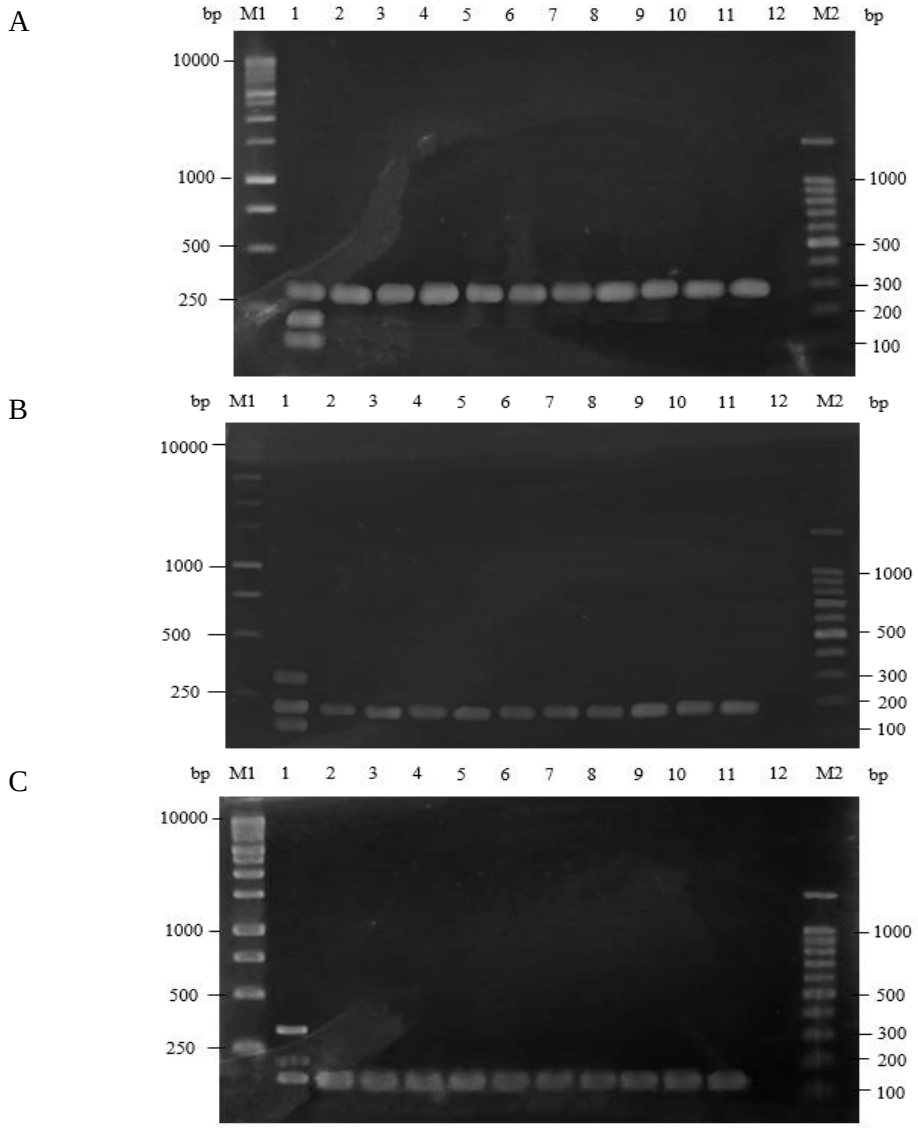

Figure 2. Gel electrophoresis of multiplex PCR for confirmation of (a) *Vibrio parahaemolyticus* isolates (297 bp; Lane 2 – 12: Isolate TA01 – TA10), (b) *Vibrio alginolyticus* isolates (199bp; Lane 2 – 12: Isolate TA11 – TA20), and (c) *Vibrio cholerae* (154 bp; Lane 2 – 12: Isolate TA21 – TA 30). Lane M1: 1 kbp DNA ladder; Lane M2: 100 bp DNA ladder; Lane 1: positive control (cocktail of *V. parahaemolyticus* ATCC 27969, *V. cholerae* KCDC 13589, and *V. alginolyticus* ATCC 17749); Lane 12: negative control

For *V. alginolyticus* isolates, eight different antibiotic resistance patterns were observed (Table 6). Among them, three isolates, namely TA24 and TA25 isolated from shrimps, and TA29 from effluent, exhibited the same patterns of P-RD-B. The MAR indices of the ten *V. alginolyticus* isolates ranged from 0.17 to 0.33. Isolate TA22, with the antibiotic resistance pattern of AMP-P-AMC-KF-IPM-B, exhibited the highest MAR index (0.33). Conversely, antibiotic resistance patterns P-RD-B, P-KF-B, and AMP-P-B displayed the lowest MAR index (0.17).

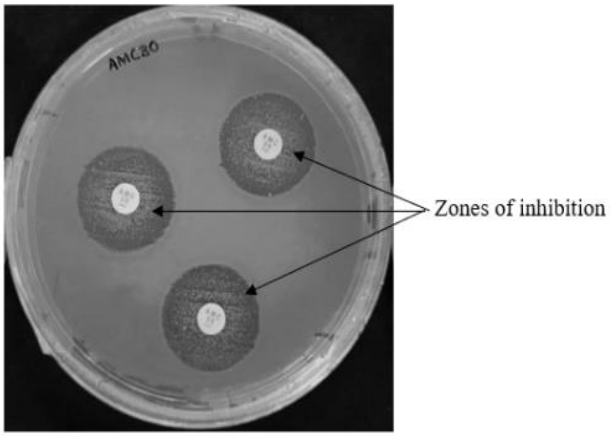

Figure 3. Colonies growth in zone of inhibition may indicate that the bacterium is resistant towards the antibiotic

Table 3. Antibiotic resistance profile of *Vibrio* spp. isolated from Telaga Air shrimp farm against 18 antibiotics

Antimicrobial Class	Antibiotic	Abbreviation	Number (%) of response of isolates to antibiotic								
			Vibrio parahaemolyticus (n=10)			*Vibrio cholera* (n=10)			*Vibrio alginolyticus* (n=10)		
			S	I	R	S	I	R	S	I	R
PenicillinS and β-lactam/ β-lactamase inhibitor combinations	Ampicillin	AMP10	2 (20)	5 (50)	3 (30)	6 (60)	2 (20)	2 (20)	6 (60)	1 (10)	3 (30)
	Amoxicillin-clavulanate	AMC30	10 (100)	-	-	10 (100)	-	-	9 (90)	-	1 (10)
	Penicillin G	P10	-	-	10 (100)	-	-	10 (100)	-	-	10 (100)
Cephalosporins/Cephems	Ceftazidime	CAZ30	9 (90)	1 (10)	-	10 (100)	-	-	10 (100)	-	-
	Cephalothin	KF30	-	3 (30)	7 (70)	2 (20)	1 (10)	7 (70)	4 (40)	-	6 (60)
Carbapenems	Imipenem	IPM10	8 (80)	-	2 (20)	6 (60)	-	4 (40)	2 (20)	5 (50)	3 (30)
	Meropenem	MEM10	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
Aminoglycosides	Amikacin	AK30	3 (30)	3 (30)	4 (40)	9 (90)	1 (10)	-	10 (100)	-	-
	Gentamicin	CN10	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
	Kanamycin	K30	3 (30)	6 (60)	1 (10)	7 (70)	3 (30)	-	8 (80)	2 (20)	-
Tetracyclines	Tetracycline	TE30	9 (90)	1 (10)	-	10 (100)	-	-	10 (100)	-	-
Quinolones	Nalidixic acid	NA30	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
	Norfloxacin	NOR10	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
Fluoroquinolones	Ciprofloxacin	CIP5	5 (50)	5 (50)	-	10 (100)	-	-	10 (100)	-	-
Phenicols	Chloramphenicol	C30	10 (100)	-	-	10 (100)	-	-	10 (100)	-	-
Macrolides	Erythromycin	E15	4 (40)	6 (60)	-	2 (20)	7 (70)	1 (10)	2 (20)	8 (80)	-
Ansamycins	Rifampicin	RD5	2 (20)	5 (50)	3 (30)	-	1 (10)	9 (90)	2 (20)	2 (20)	6 (60)
Polypeptides	Bacitracin	B10	-	-	10 (100)	2 (20)	-	8 (80)	-	-	10 (100)

Table 4. The antibiotic resistance profile patterns and multiple antibiotic resistance (MAR) indices of *V. parahaemolyticus* isolates

Isolate	Source	Antibiotic resistance profiles*	MAR index
TA01	Water	PB	0.11
TA02	Water	PAKKFB	0.22
TA03	Water	PAKKFB	0.22
TA04	Sediment	AMPPKFRDB	0.28
TA05	Sediment	PKFRDB	0.22
TA06	Sediment	AMPPAKKFKB	0.33
TA07	Shrimp	AMPPAKKFRDIPMB	0.39
TA08	Shrimp	PAKB	0.17
TA09	Effluent	PKFIPMB	0.22
TA10	Effluent	PB	0.11

*Antibiotics: Ampicillin (AMP); Penicillin G (P); Amoxicillin-clavulanate (AMC); Amikacin (AK); Erythromycin (E); Cephalothin (KF); Rifampicin (RD); Imipenem (IPM); Kanamycin (K); Bacitracin (B)

Table 5. The antibiotic resistance profile patterns and multiple antibiotic resistance (MAR) indices of *V. cholerae* isolates

Isolate	Source	Antibiotic resistance profiles*	MAR index
TA11	Water	PKFRDIPMB	0.28
TA12	Water	PKFRDIPMB	0.28
TA13	Water	AMPPEKFRDB	0.33
TA14	Sediment	AMPPKFRDB	0.28
TA15	Sediment	PKFRDIPM	0.22
TA16	Sediment	PRDB	0.17
TA17	Shrimp	PKFRD	0.17
TA18	Shrimp	PRDB	0.17
TA19	Effluent	PKFRDIPMB	0.28
TA20	Effluent	PB	0.11

*Antibiotics: Ampicillin (AMP); Penicillin G (P); Amoxicillin-clavulanate (AMC); Amikacin (AK); Erythromycin (E); Cephalothin (KF); Rifampicin (RD); Imipenem (IPM); Kanamycin (K); Bacitracin (B)

Table 6. The antibiotic resistance profile patterns and multiple antibiotic resistance (MAR) indices of *V. alginolyticus* isolates

Isolate	Source	Antibiotic resistance profiles*	MAR index
TA21	Shrimp	AMPPKFRDB	0.28
TA22	Shrimp	AMPPAMCKFIPMB	0.33
TA23	Shrimp	PKFIPMB	0.22
TA24	Shrimp	PRDB	0.17
TA25	Shrimp	PRDB	0.17
TA26	Effluent	PKFRDB	0.22
TA27	Effluent	PKFB	0.17
TA28	Effluent	PKFRDIPMB	0.28
TA29	Effluent	PRDB	0.17
TA30	Effluent	AMPPB	0.17

*Antibiotics: Ampicillin (AMP); Penicillin G (P); Amoxicillin-clavulanate (AMC); Amikacin (AK); Erythromycin (E); Cephalothin (KF); Rifampicin (RD); Imipenem (IPM); Kanamycin (K); Bacitracin (B)

Detection of Virulence Genes in *Vibrio* species

Virulence genes detection was performed on all 30 *Vibrio* spp. isolates by using primer sets that target the specific virulence gene sequences. Table 7 below summarised the results of the PCR where presence of specific bands was observed and recorded. PCR was conducted on ten *V. parahaemolyticus* isolates to detect all virulence genes listed in Table 1.

Based on the PCR results, the presence of *tlh*, *toxS*, and *toxR* genes was observed in all ten *V. parahaemolyticus* isolates (Table 7). In Figure 4 below, a band with a size of 450 bp was detected in all *V. parahaemolyticus* isolates, consistent with the expected size of the *tlh* gene. However, no bands corresponding to the expected sizes of *trh* and *tdh* genes were identified. In Figure 5, two bands were observed in each isolate, with sizes of 640 bp and 596 bp, matching the

anticipated sizes of *toxS* and *toxR*, respectively. Meanwhile, the detection of the *pirA* gene, which contributes to some strains of *V. parahaemolyticus* the ability to cause Acute Hepatopancreatic Necrosis Disease (AHPND), yielded negative results for all *V. parahaemolyticus* isolates (Figure 6).

Meanwhile, in the *V. cholerae* isolates, four virulence genes were positively detected in all ten isolates. As depicted in Figure 7 below, two bands with sizes of 640 bp and 595 bp, corresponding to the expected sizes of the *toxS* and *toxR* genes, respectively, were observed for each isolate. Moreover, in Figure 8, two positive bands corresponding to the *rtxA* (417 bp) and *rtxC* (263 bp) genes were observed for each *V. cholerae* isolate, while *ctxB* (460 bp) was not detected in any of the isolates.

No positive detection of the nine targeted virulence genes was observed in all *V. alginolyticus* isolates. As depicted in Figure 9, no bands were detected for all ten isolates in the detection of the *toxR* and *toxS* genes (similar results were observed with the other virulence genes). This indicates that none of the isolated *V. alginolyticus* harbour the targeted virulence genes.

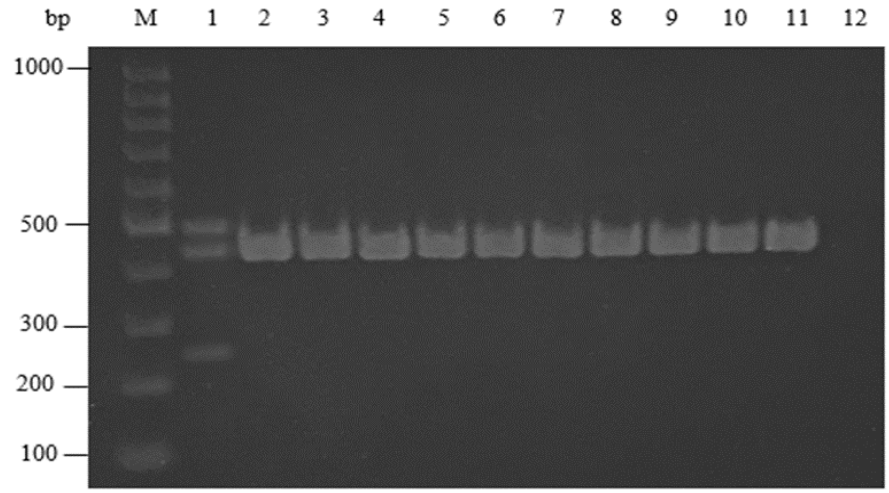

Figure 4. PCR result for the detection of *tdh* (269 bp), *trh* (500 bp), and *tlh* (450 bp) virulence genes in *V. parahaemolyticus* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. parahaemolyticus* TA01 – TA10; lane 12: negative control (sterile dH_2O).

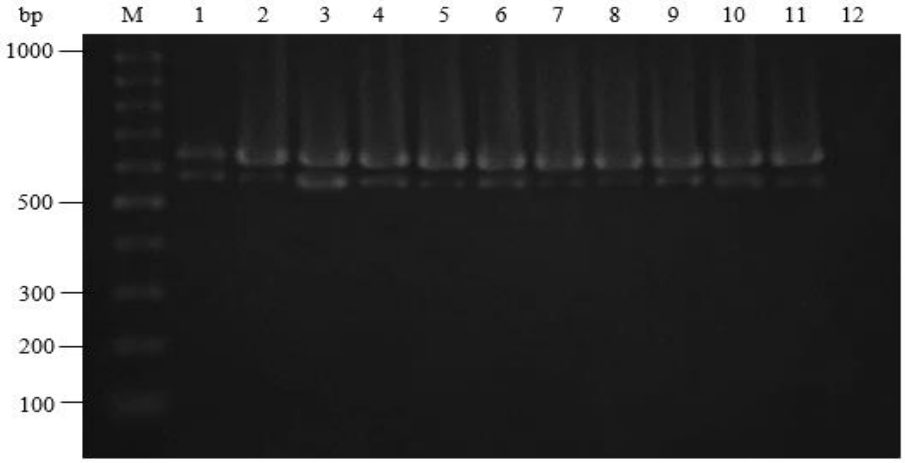

Figure 5. PCR result for the detection of *toxR* (596 bp) and *toxS* (640 bp) virulence genes in *V. parahaemolyticus* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. parahaemolyticus* TA01 – TA10; lane 12: negative control (sterile dH_2O).

Table 7. Detection of virulence genes in *Vibrio* spp. isolates using PCR

Vibrio spp.	Isolate	Virulence gene								
		tdh	*trh*	*tlh*	*toxS*	*toxR*	*toxA*	*rtxA*	*rtxC*	*ctxB*
Vibrio parahaemolyticus	TA01	-	-	+	+	+	-	-	-	-
	TA02	-	-	+	+	+	-	-	-	-
	TA03	-	-	+	+	+	-	-	-	-
	TA04	-	-	+	+	+	-	-	-	-
	TA05	-	-	+	+	+	-	-	-	-
	TA06	-	-	+	+	+	-	-	-	-
	TA07	-	-	+	+	+	-	-	-	-
	TA08	-	-	+	+	+	-	-	-	-
	TA09	-	-	+	+	+	-	-	-	-
	TA10	-	-	+	+	+	-	-	-	-
Vibrio cholera	TA11	-	-	-	+	+	-	+	+	-
	TA12	-	-	-	+	+	-	+	+	-
	TA13	-	-	-	+	+	-	+	+	-
	TA14	-	-	-	+	+	-	+	+	-
	TA15	-	-	-	+	+	-	+	+	-
	TA16	-	-	-	+	+	-	+	+	-
	TA17	-	-	-	+	+	-	+	+	-
	TA18	-	-	-	+	+	-	+	+	-
	TA19	-	-	-	+	+	-	+	+	-
	TA20	-	-	-	+	+	-	+	+	-
Vibrio alginolyticus	TA21	-	-	-	-	-	-	-	-	-
	TA22	-	-	-	-	-	-	-	-	-
	TA23	-	-	-	-	-	-	-	-	-
	TA24	-	-	-	-	-	-	-	-	-
	TA25	-	-	-	-	-	-	-	-	-
	TA26	-	-	-	-	-	-	-	-	-
	TA27	-	-	-	-	-	-	-	-	-
	TA28	-	-	-	-	-	-	-	-	-
	TA29	-	-	-	-	-	-	-	-	-
	TA30	-	-	-	-	-	-	-	-	-

(+): positive detection; (-): no detection

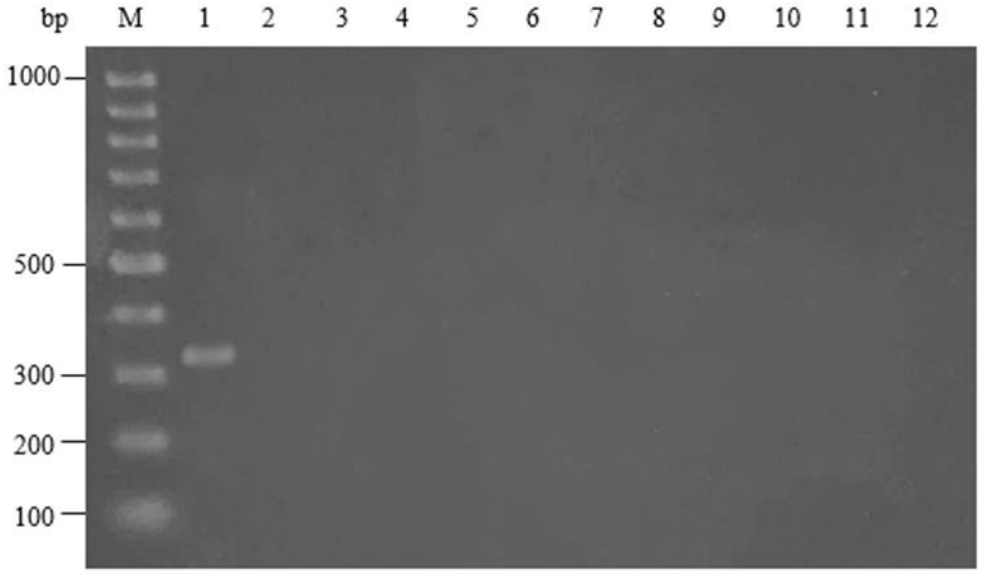

Figure 6: PCR result for the detection of pirA (333 bp) virulence genes in *V. parahaemolyticus* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. parahaemolyticus* TA01 – TA10; lane 12: negative control (sterile dH_2O)

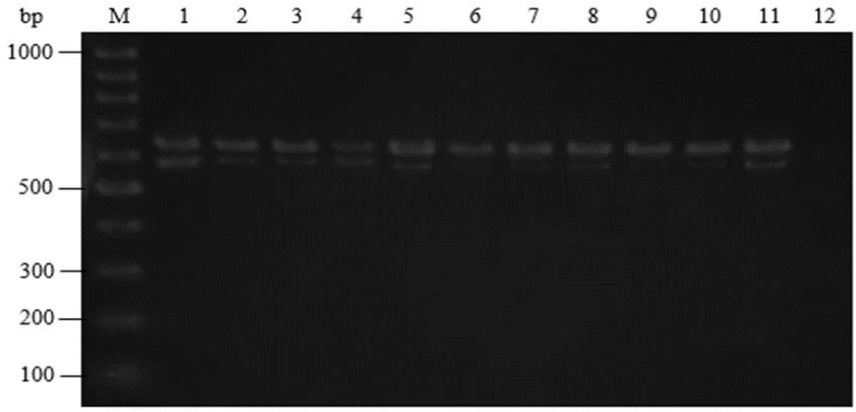

Figure 7. PCR result for the detection of *toxR* (595 bp) and *toxS* (640 bp) virulence genes in *V. cholerae* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. cholerae* TA11 – TA20; lane 12: negative control (sterile dH_2O)

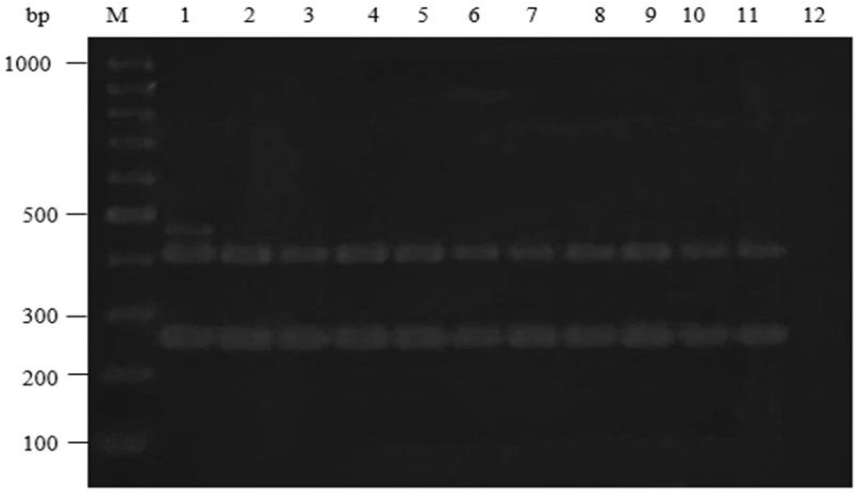

Figure 8. PCR result for the detection of *rtxA* (417 bp), *rtxC* (263 bp) and *ctxB* (460 bp) virulence genes in *V. cholerae* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. cholerae* TA11 – TA20; lane 12: negative control (sterile dH_2O)

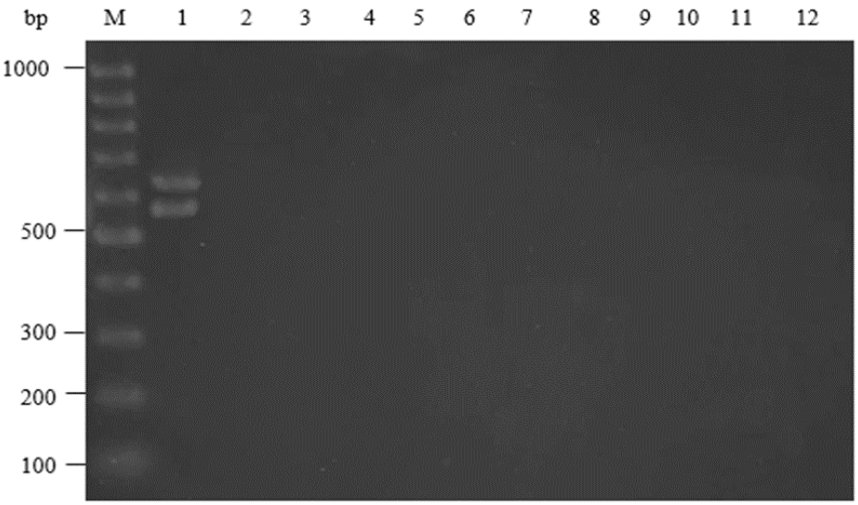

Figure 9. PCR result for the detection of *toxR* (595 bp) and *toxS* (640 bp) virulence genes in *V. alginolyticus* isolates viewed in 1.5% agarose gel. Lane M: 100 bp DNA ladder; lane 1: positive control; lane 2 – 11: *V. alginolyticus* TA21 – TA30; lane 12: negative control (sterile dH_2O)

DISCUSSION

The antibiotic susceptibility test revealed that all 30 *Vibrio* spp. isolates exhibited multiple resistance to at least two antibiotics. These findings parallel those of an antibiotic resistance study conducted in cultured shrimp farms in the Philippines, where *Vibrio* spp. isolated from ponds employing antibiotics displayed resistance to multiple antibiotics (Tendencia & De la Peña, 2001; Elmahdi *et al*., 2016). The MAR indices of the *Vibrio* spp. isolated from Telaga Air shrimp farm ranged from 0.11 to 0.39, with the majority (60%) of the total isolates having MAR indices exceeding 0.2. Our findings align with those of Devadas *et al*. (2015), who reported that 63.8% of zoonotic bacteria, including *V. parahaemolyticus*, isolated from shrimp aquaculture environments in Selangor, exhibited MAR indices greater than 0.2. Our finding suggests that these isolates likely originated from environments with considerable antibiotic pressure, consistent with aquaculture systems where prophylactic or therapeutic antibiotic usage is prevalent. Despite the farm manager's assertion that antibiotic treatment was not administered in the Telaga Air shrimp farm, the presence of isolates with MAR indices exceeding 0.2 suggests they may have originated from a contaminated source with extensive antibiotic use. This suggests the existence of residual antibiotics originating from wastewater or nearby agricultural and aquaculture activities (Hanna *et al*., 2023). Additionally, certain antibiotics demonstrate stability and can persist in the environment long after their use has ceased, leading to contamination by the accumulated residual antibiotics (Larsson, 2014; Lundborg & Tamhankar, 2017).

The implications of MAR > 0.2 in aquaculture are significant. It not only suggests ongoing antibiotic pressure in the farming environment but also indicates potential public health risks through the food chain and the surrounding ecosystem. Resistant *Vibrio* strains may transfer resistance genes to other environmental or pathogenic bacteria via horizontal gene transfer. Moreover, the presence of high MAR index values in isolates from water, sediment, and shrimp samples underscores the widespread nature of this resistance across various environmental compartments within the shrimp farm. From the standpoint of AMR dissemination, aquaculture alarmingly plays a significant role in the emergence, persistence, and transmission of resistance factors within the aquatic environment. The environment, as a whole, serves as a vast reservoir of antibiotic resistance genes (ARGs), with the aquatic environment, in particular, serving as a crucial reservoir of mobile genetic elements (MGEs) carrying these ARGs. Hence, the aquatic environment serves as a common ground for the transmission of antimicrobial resistance between bacteria (Salgueiro *et al*., 2024). Moreover, the boundaries between species in aquatic environments are narrow due to the genomic plasticity of *Vibrio* species. Horizontal gene transfer between them, even between the environmental and clinical strains, are frequent events (Liang *et al*., 2022).

Overall, shrimp-derived isolates, particularly from *V. parahaemolyticus* and *V. alginolyticus*, tend to exhibit higher MAR indices compared to those from water, sediment, or effluent, suggesting greater antibiotic selective pressure in host-associated environments. This pattern may suggest increased selective pressure for antibiotic resistance in host-associated bacteria, possibly due to antibiotic use in aquaculture or accumulation of residues in shrimp tissues (Luu *et al*., 2021). This notion is further supported by a study by Sharma *et al*. (2021), which found that farm-raised shrimp—fed artificially formulated diets and raised in a shared aquatic microbiota—tend to harbour a higher prevalence of ARGs in their guts compared to wild-caught shrimp. Evidently, chitin, the predominant structural component of shrimp and other crustacean exoskeletons, has been identified as a potential intermediary surface facilitating the proliferation and dissemination of AMR through biofilm attachment, as highlighted by Meibom *et al*. (2005). Future investigations employing molecular typing and resistance gene profiling would provide deeper insights into the relationships and mechanisms underpinning these resistance patterns.

Numerous studies have highlighted the high resistance of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* to ampicillin (Oh *et al*., 2011; Elmahdi *et al*., 2016; Abioye *et al*., 2023). Al-Othrubi *et al*. (2014) reported that over 50%

of *V. parahaemolyticus* isolated from cockles and shrimp samples from various Malaysian markets displayed high resistance to ampicillin. Similarly, Amalina *et al*. (2019) found that the majority of *Vibrio* spp. isolated from cultured groupers in farms across Peninsular Malaysia exhibited high resistance to ampicillin. However, in this investigation, only 30% of *V. parahaemolyticus* and *V. alginolyticus* isolates, and 20% of *V. cholerae* isolates, exhibited resistance to ampicillin, with the remaining isolates displaying susceptibility or intermediate resistance to the antibiotic. Notably, *V. parahaemolyticus* exhibited a higher proportion of isolates with intermediate resistance (50%) compared to the other two *Vibrio* spp., indicating that the dosage of the ampicillin antibiotics needs to be adjusted to treat infection caused by some of the *V. parahaemolyticus* strains (CDC, 2019b). Overall, only a small percentage of isolates from the Telaga Air shrimp farm displayed resistance to ampicillin.

In this investigation, antibiotics belonging to specific classes remained highly effective at controlling all *Vibrio* spp. isolates, including phenicols (Chloramphenicol), tetracyclines (Tetracycline), cephalosporins (Ceftazidime), fluoroquinolones (Ciprofloxacin), quinolones (Norfloxacin and Nalidixic acid), aminoglycosides (Gentamicin), and carbapenems (Meropenem). These antibiotics operate through diverse mechanisms to combat bacterial infections. Phenicol, tetracycline, and aminoglycoside antibiotics hinder protein synthesis, with phenicols targeting the 50s ribosomal subunit, and tetracyclines and aminoglycosides affecting the 30s ribosomal subunit (Kapoor *et al*., 2017). Notably, tetracycline is often recommended for treating severe *Vibrio* spp. infections due to its capacity to impede the synthesis of pathogenic extracellular enzymes, particularly proteases and lipases (Elmahdi *et al*., 2016). Meanwhile, cephalosporins and carbapenems inhibit cell wall synthesis or function, thereby disrupting bacterial growth and multiplication (Kapoor *et al*., 2017). Additionally, fluoroquinolones and quinolones hinder DNA replication and synthesis, and impede cell division by interfering with the function of DNA gyrase and topoisomerases (Hooper & Jacoby, 2016; Correia *et al*., 2017).

In contrast, both Penicillin G and Bacitracin were found to be completely ineffective against the *Vibrio* spp. isolates in this study. Resistance of *Vibrio* spp. to these antibiotics has been documented in several previous studies. For instance, Amalina *et al*. (2019) reported that 82% of *Vibrio* spp. isolated from cultured grouper in Peninsular Malaysia exhibited resistance to Penicillin G, while over 50% of the isolates were also resistant to Bacitracin. Furthermore, Sahilah *et al*. (2014) found that 98% of *V. parahaemolyticus* isolated from cockles along the east coast of Peninsular Malaysia showed resistance to Bacitracin. These variations in resistance patterns, including those observed in the present study, suggest that geographical differences may play a significant role in shaping antibiotic resistance profiles among *Vibrio* species (Amalina *et al*., 2019). Besides, these antibiotics have been used for a long time in the industry in Malaysia, thus supporting the claim that continuous exposure to antibiotics will cause bacteria to develop resistance to them (Kathleen *et al*., 2016; Amalina *et al*., 2019)

Bacteria, especially pathogenic strains, often undergo natural genetic changes over time, leading to their development of antimicrobial resistance. However, rapid emergence and dissemination of these multidrug-resistant (MDR) strains are exacerbated by human activities, particularly through the excessive use and misuse of antimicrobials in humans, animals, and plants (WHO, 2023). The spread of resistance genes among bacteria in aquatic environments can occur swiftly through horizontal gene transfer. Several studies have documented the discovery of resistance genes in clinical isolates of *Vibrio* spp. that were previously identified in environmental isolates, suggesting genetic exchange between clinical and environmental strains (Elmahdi *et al*., 2016).

The isolates in this study underwent screening to detect the presence of virulence genes alongside assessing their susceptibility to antibiotics. The detection of the *tlh* gene in all *V. parahaemolyticus* isolates, contrasted with its absence in the *V. cholerae* and *V. alginolyticus* isolates, suggests high conservation of this gene in *V. parahaemolyticus*, often utilised as a signature molecular marker for the species (Gutierrez West *et al*., 2013). Nevertheless, several studies have indicated the presence of the

tlh gene in other *Vibrio* spp. apart from *V. parahaemolyticus*. As reported by Wang *et al*. (2007), the *tlh* gene is widespread in various *Vibrio* spp., including *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, and *V. anguillarum* (Yáñez *et al*., 2015). Consequently, the *tlh* gene may not be as exclusive to *V. parahaemolyticus* as previously assumed, potentially diminishing its precision as a species-specific marker.

Most pathogenic strains of *V. parahaemolyticus* typically possess the *tdh* and/or *trh* genes alongside the *tlh* genes (Yáñez *et al*., 2015). In clinical isolates, the *tdh* gene was detected in the majority (95%) of cases, highlighting thermostable direct hemolysin (TDH) as one of the main virulence factors produced by *V. parahaemolyticus* (Liu, 2003; Wang *et al*., 2015). The absence of both *tdh* and *trh* genes, which encode TDH and its homolog, TDH-related hemolysin (TRH), respectively, in all isolates may suggest that these isolates are non-pathogenic or have low pathogenicity in humans, as these two genes are commonly utilised as indicators of *V. parahaemolyticus* pathogenicity (Bej *et al*., 1999; Gutierrez West *et al*., 2013). Our findings are consistent with numerous studies indicating that the majority of environmental strains of *V. parahaemolyticus* are non-pathogenic, with the *tdh* and/or *trh* genes detected only infrequently in these strains (Caburlotto *et al*., 2009). However, the incidence of pathogenic *Vibrio* spp. continues to rise annually, resulting in an increase in infection cases (Elmahdi *et al*., 2016). There are also evidences suggesting that pathogenic *V. parahaemolyticus* acquired *tdh* genes through horizontal gene transfer (Okuda *et al*., 2001).

While the precise roles of the *tlh* gene in *V. parahaemolyticus* pathogenicity remain unclear, it encodes for phospholipase A2 and thermolabile hemolysin, both of which have been reported to induce lysis of human erythrocytes (Shinoda *et al*., 1991; Wang *et al*., 2015). Furthermore, its expression has been demonstrated to be upregulated in environments resembling those of the human intestines, suggesting its involvement in the adaptation, survival, and virulence of *V. parahaemolyticus* within the host gastrointestinal tract (Gotoh *et al*., 2010; Yáñez *et al*., 2015). This notion is further supported by findings indicating that HeLa, Chang liver, and RAW264.7 cells treated with purified TLH protein exhibit severe cytotoxicity, which is dose and time-dependent, thereby suggesting that TLH shares similar biological functions with the TDH and TRH toxins (Wang *et al*., 2012; Wang *et al*., 2015). Therefore, despite the unclear roles of TLH proteins in *V. parahaemolyticus* pathogenicity, it is premature to rule out these *V. parahaemolyticus* isolates as non-pathogenic simply because the *tdh* and *trh* genes were not detected. Moreover, some studies report that 10% of clinical isolates of *V. parahaemolyticus* lack the *tdh* and *trh* genes but still exhibit high cytotoxicity to gastrointestinal cells, suggesting that there are other virulence factors that contributed to their pathogenicity (Raghunath, 2014).

One of the diseases causing significant losses in shrimp farms is Acute Hepatopancreatic Necrosis Disease (AHPND). This disease is induced by specific strains of *V. parahaemolyticus* (VP_{AHPND}) carrying the plasmid (pVA1) containing *pirA* and *pirB* genes, encoding binary toxins PirA and PirB (Sirikharin *et al*., 2015; Dhar *et al*., 2019; Hossain *et al*., 2020). The presence of both toxin subunits is necessary for the manifestation of AHPND pathologies, and these toxins induce hepatopancreatic necrosis in infected shrimp, leading to rapid progression and 100% mortality in severely infected ponds (Sirikharin *et al*., 2015; Hossain *et al*., 2020). For *pirA* gene detection, the AP3 detection method has been widely used in previous studies and is recommended for confirming AHPND-positive strains (Hossain *et al*., 2020). Compared to other VP_{AHPND} detection methods (AP1 and AP2), the primer sets employed in the AP3 method have shown the highest sensitivity and specificity (Sirikharin *et al*., 2015; Hossain *et al*., 2020). In this study, none of the *Vibrio* spp. isolates, particularly the *V. parahaemolyticus* isolates, exhibited the presence of the *pirA* gene, indicating that these isolates are not AHPND-causing strains. However, continuous monitoring is essential as there have been reported cases where the acquisition of *pirA* and *pirB* genes through horizontal gene transfer has transformed non-AHPND strains into VP_{AHPND} strains (Dhar *et al*., 2019).

In all *V. cholerae* isolates, two out of three virulence genes typically associated with pathogenicity were detected: the *rtxA* and *rtxC* genes belonging to the repeat in toxin (RTX)

family. This family encompasses gene clusters including *rtxA*, encoding a putative cytotoxin, *rtxC*, encoding an acyltransferase, and *rtxB* and *rtxD*, involved in producing proteins responsible for toxin transportation (Chow *et al*., 2001). Chow *et al*. (2001) demonstrated a concurrent relationship between positive detection of *rtxA* and *rtxC* genes and positive results in HEp-2 cytotoxicity assays in clinical and environmental *V. cholerae* isolates, and vice versa. This finding demonstrated the integrity between the genotypic and phenotypic expressions of these genes across strains. Supporting this association, strains with deleted RTX gene clusters showed negative results in PCR detection and cytotoxicity assays (Chow *et al*., 2001). Therefore, the presence of both *rtxA* and *rtxC* in *V. cholerae* isolates from the Telaga Air shrimp farm indicates their pathogenic potential.

Additionally, the *ctxB* gene is a fundamental component that encodes the cholera toxin (CT), responsible for cholera syndrome characterised by acute diarrheal diseases (Chow *et al*., 2001; Takahashi *et al*., 2021). CT is regarded as the primary virulence factor in *V. cholerae* and is frequently associated with strains of significant pandemic potential (Chow *et al*., 2001). All *V. cholerae* isolates in this study lacked the *ctxB* gene. According to Chow *et al*. (2001), non-epidemic *V. cholerae* non-O1 serogroup strains that are positive for *rtxA* and *rtxC* but negative for *ctxB* only caused sporadic, milder cases of diarrhoea. However, these strains should not be underestimated as RTX cytotoxins can still induce necrosis and inflammation in the gastrointestinal tract (Chow *et al*., 2001; Kim *et al*., 2012).

The primers used for targeting the *toxR* and *toxS* genes used in this study (Table 1) were originally used for targeting *V. cholerae*-associated *toxR* and *toxS* genes. However, subsequent studies revealed that the *toxR* gene is also present in other *Vibrio* spp., including *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, *V. fluvialis*, and *V. vulnificus*, with homologous sequences observed in the internal portion of the nucleotide sequences of the *toxR* genes of at least three different *Vibrio* species (Okuda *et al*., 2001; Wang *et al*., 2015). As such, *toxR* and its integral membrane binding partner, *toxS* genes were also detected in all *V. parahaemolyticus* isolates in addition to all *V. cholerae* isolates in this study. Unlike the *tdh*, *trh*, and *tlh* gene in *V. parahaemolyticus* and *rtxA*, *rtxC*, and *ctxB* genes in *V. cholerae*, *toxR* and *toxS* are not directly related to the expression of virulence factors. Instead, they encode integral membrane proteins, ToxR and ToxS, that activate the transcription of virulence genes in the *Vibronaceaea* family (Pfau and Taylor, 1998; Lembke *et al*., 2020). The *toxR* gene was initially discovered as a regulatory gene for the *ctx* gene encoding CT in *V. cholerae*. The presence of these genes in *V. parahaemolyticus* isolates suggests that they may have acquired these exogenous genes through horizontal gene transfer (Sechi *et al*., 2000). Indeed, a study by Lin *et al*. (1993) showed that *V. parahaemolyticus*-associated ToxR protein is highly similar to the ToxR protein produced by *V. cholerae*.

It was later discovered that *toxR* gene plays a multifaceted role and is not only responsible to regulate the expression of virulence genes, but also in coordinating the regulation of multiple genes that can affect the survival and adaptability of *Vibrio* spp. in various environmental conditions (Okuda *et al*., 2001). For example, *toxR* was initially known to promote the expression of *tdh* gene with the presence of *toxS* gene in *V. parahaemolyticus* (Lin *et al*., 1993; Zhang *et al*., 2018). However, since *tdh* gene was not detected in the *V. parahaemolyticus* isolates in this study, the detection of both *toxR* and *toxS* genes in all isolates suggests that these genes play other roles that contribute to the adaptability and survival of the isolates. Similarly, the absence of *ctxB* gene in the *V. cholerae* isolates suggested that the *toxR* and *toxS* gene that were detected in them plays role other than the expression of the *ctx* virulence genes.

Contrary to the initial belief that the primary roles of the *toxR* and *toxS* genes are to stimulate the expression of virulence genes in the *Vibronaceae* family, a study by Okuda *et al*. (2001) demonstrated that the original function of these genes was to modulate the expression of outer membrane proteins (OMP)-encoding genes for bacterial adaptation in various environmental conditions. ToxR-modulated expression of OMPs was shown to contribute to the ability of *Vibrio* spp. to survive in the gastrointestinal tract by facilitating colonisation, stimulating the expression of virulence factors, and conferring bile resistance (Okuda *et al*.,

2001; Lembke *et al*., 2020). These findings lend support to the results obtained in this study, suggesting that the presence of *toxR* and *toxS* genes in the isolates may play a more significant role in the adaptability of the *Vibrio* spp. rather than in the expression of their virulence genes, especially considering the absence of associated virulence genes.

Moreover, several studies have indicated the involvement of the *toxR* gene in the regulation of biofilm formation. The expression of ToxR protein has been reported to be influenced by cell density and is implicated in the quorum sensing (QS) mechanism of *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. anguillarum*, which can coordinate biofilm formation in response to environmental cues (Chen *et al*., 2018; Zhang *et al*., 2018; Zhang *et al*., 2021). A study conducted by Chen *et al*. (2018) suggested that ToxR plays a pivotal role in biofilm formation in *V. parahaemolyticus*. The reduced biofilm formation observed in a *toxR* mutant strain compared to the wild-type *V. parahaemolyticus* indicates the involvement of ToxR in promoting biofilm production. In addition, the restoration of biofilm formation in the complemented mutant strain further supports the role of ToxR in this process (Chen *et al*., 2018). Therefore, ToxR likely regulates genes or pathways that contribute to the formation of mature biofilms, thereby enhancing the adaptability and pathogenicity of *V. parahaemolyticus* (Gao *et al*., 2022).

In all *V. alginolyticus* isolates, none of the tested virulence genes were detected. Despite the *toxR* gene being commonly reported in many studies as the second most frequently detected virulence gene in *V. alginolyticus* after the *ompK* gene, no positive detections were observed in any *V. alginolyticus* isolates from the Telaga Air shrimp farm (Abd Wahid *et al*., 2022). Additionally, the *tlh* genes associated with *V. parahaemolyticus*, which were reportedly found in all seven *V. alginolyticus* strains from shrimp hatcheries in Southern China as reported by Xue *et al*. (2022), were not detected in all *V. alginolyticus* isolates in this study (Abd Wahid *et al*., 2022). The absence of other virulence genes originally from various *Vibrio* spp. in all isolates suggests a low frequency of gene transfer events between *V. alginolyticus* and other *Vibrio* spp. in the shrimp farm or few strains are retaining these genes through horizontal gene transfer mechanisms (Xue *et al*., 2022).

While this study primarily focused on the phenotypic and genotypic characterization of antibiotic resistance and the presence of selected virulence genes in *Vibrio* spp. isolates, the potential relationship between these two factors is of significant interest. Although no formal analysis was conducted to examine correlations between multidrug resistance and the presence of virulence genes in this study, the literature suggests that the relationship between antibiotic resistance and virulence traits can be complex. Previous studies have shown that antibiotic resistance and virulence genes can be independently acquired and maintained in bacterial populations (Beceiro *et al*., 2013). However, there are instances where both traits may be co-selected or linked under certain environmental conditions or selective pressures. Factors such as bacterial species, the mechanisms of resistance and virulence, regulatory networks, ecosystems and environmental factors, such as antibiotic use in aquaculture, may influence the presence and expression of both resistance and virulence traits (Cepas & Soto, 2020).

Several studies have reported conflicting findings regarding the relationship between antimicrobial resistance and virulence. For example, strains of uropathogenic *E. coli* (UPEC) that exhibited resistance to quinolones were found to possess fewer virulence factors or showed decreased expression of virulence genes associated with the development of cystitis and pyelonephritis in the urinary tract (Vila *et al*., 2002). Similarly, an inverse relationship between antibiotic resistance and virulence was observed in Non-O1, Non-O139 *V. cholerae* isolates, reportedly due to a fitness trade-off (Abioye *et al*., 2023). In contrast, increased virulence linked to the development of swarming motility in *Pseudomonas aeruginosa* has been associated with increased resistance to polymyxin B, ciprofloxacin, and gentamicin (Beceiro *et al*., 2013). However, another study reported no correlation between biofilm formation, virulence factors, and antibiotic resistance in *P. aeruginosa*, thereby contradicting the previous findings (Gajdács *et al*., 2021). These discrepancies highlight the complex and multifactorial nature of the relationship between antibiotic resistance and

virulence in bacteria, which can vary depending on the species, genetic background, and environmental conditions. Further investigation, including more extensive analyses of the genomic and environmental factors influencing these traits, is essential to fully understand the interplay between antibiotic resistance and virulence of the *Vibrio* isolates in this study.

In order to curtail the dissemination of MAR bacterial strains and prevent the transfer of virulence genes among bacteria within shrimp farms, employing prudent practices is imperative. Farmers must prioritise the cultivation and provision of specific pathogen-free (SPF) shrimp stocks, a measure proven to enhance shrimp production across numerous countries (Nillian *et al*., 2022). However, effective disease control necessitates strict management protocols encompassing physical barriers, meticulous water treatment control, carrier exclusion strategies, and vigilant feed management systems (Nillian *et al*., 2022). Furthermore, regular monitoring of *Vibrio* spp. prevalence and antibiotic resistance patterns within farms assumes paramount importance, serving as a pivotal precursor to informed management decisions.

CONCLUSION

This study has provided valuable insights into the antibiotic resistance profiles and virulence gene profiles of *Vibrio* spp. isolates obtained from the Telaga Air shrimp farm in Kuching, Sarawak. The primary objectives of the study were to assess the antibiotic resistance patterns and the presence of virulence genes in *Vibrio* spp. isolates, and to evaluate their potential public health risk. The findings indicate widespread antibiotic resistance among *Vibrio* spp. isolates, likely attributed to the extensive use of antibiotics or the presence of their residuals in aquaculture settings. Additionally, the presence of isolates with MAR indices higher than 0.2 suggests potential contamination from sources with high antibiotic usage, such as wastewater or nearby agricultural and aquaculture activities. The study also identified significant diversity in the virulence gene profiles among *Vibrio* spp. isolates. While some isolates exhibited key virulence genes associated with pathogenicity, others lacked these genes, indicating variability in their pathogenic potential. These findings highlight the importance of continuous testing and monitoring to mitigate the risks of antibiotic resistance and pathogenic *Vibrio* spp. in shrimp farming environments.

However, despite the findings obtained in this study, the surveillance data is only limited to the strains isolated from the Telaga Air shrimp farm, and hence, do not represent the comprehensive epidemiology in Sarawak. Moreover, given the high occurrence of horizontal gene transfer within bacterial populations, ongoing and a more comprehensive monitoring is essential to ensure that limited spread of virulence genes occurs between pathogenic and non-pathogenic strains. Additionally, monitoring antibiotic usage and bacterial resistance patterns is crucial for effective disease management and sustainable aquaculture practices.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest in preparing this article.

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