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Diversity of *Vibrio* spp. as pathogenic bacteria and their antimicrobial resistance profile isolated from *Penaeus vannamei* ponds

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Abstract. *Amalia R, Windarto S, Sibero MT, Saputra D. 2024. Diversity of* Vibrio *spp. as pathogenic bacteria and their antimicrobial resistance profile isolated from* Penaeus vannamei *ponds. Biodiversitas 25: 4450-4461.* Vibriosis is one of the most severe diseases affecting brackish aquaculture systems. The objective of this study was to investigate the diversity of unspeciated *Vibrio* spp. and antimicrobial resistance patterns of pathogenic *Vibrio* sp. bacteria isolated from *Penaeus vannamei* Boone 1931 ponds. Water and sediment samples were collected from several intensive ponds at the Marine Science Techno Park (MSTP), Universitas Diponegoro, Jepara, Indonesia. The result showed that the total vibrio count (TVC) ranged from 0.46×10^7 to 7.07×10^7 CFU mL^{-1} in water samples and from 4.73×10^7 to 20.07×10^7 (2.01×10^8) CFU mL^{-1} in sediment samples. In total, 150 bacterial cultures were successfully isolated. Furthermore, yellow, greenish-yellow, green, and dark green bacterial colonies were observed on the TCBS agar. Four out of the 150 isolates bacteria, T3.S.1, T1.B.1, T2.S.1, and TK.A.1 were selected based on different genetic profiles and identified by 16S rRNA sequencing. These isolates belonged to the genus *Vibrio* and were closely related to *Vibrio alginolyticus*, *Photobacterium damselae*, *Vibrio parahaemolyticus*, and *Vibrio harveyi*. The results of antibiotic resistance revealed that gentamicin (Cn); ampicilin (Amp); ciprofloxacin (Cip); tetracycline (Te); chloramphenicol (C); and amoxcicilin (Aml) were ineffective against *V. alginolyticus*, *P. damselae*, *V. parahaemolyticus*, and *V. harveyi*, finding indicates that these bacteria were resistant to antibiotics. Only *P. damselae* showed intermediate resistance to chloramphenicol (C). Further research is necessary to detect antibiotic resistance genes and virulence genes in these isolates.

Keywords: Antibiotics, antibiotic resistance bacteria, *Penaeus vannamei* ponds, *Vibrio* sp., 16S rRNA

INTRODUCTION

Penaeus vannamei Boone 1931 is considered to have high potential for intensive aquaculture worldwide. However, over-intensive cultivation to boost production has led to severe bacterial disease outbreaks in aquaculture (Ina-Salwany 2019; Deng et al. 2020). The Food and Agriculture Organization (FAO) estimates that global aquaculture losses due to disease outbreaks amount to $6 billion annually (FAO 2022). This is one of the most significant barriers to fisheries output, particularly for vannamei shrimp. The most common pathogens in shrimp farms include viruses, bacteria, protozoa, fungi, and metazoan parasites (Sriurairatana et al. 2014; Mastan 2015; Amatul-Samahah et al. 2020).

Vibriosis is the most common bacterial disease affecting *P. vannamei* (Amatul-Samahah et al. 2020). These bacteria significantly increase the risk of shrimp culture and environmental transmission of pathogens (Yu et al. 2023) and pose a real threat to the sustainable production of shrimp culture. Several studies have shown that vibriosis infection can cause mortalities up to 100% in all shrimp stages, from nauplius, zoea, mysis, and post-larvae to adults in rearing ponds (Kumar et al. 2014; Yuhana and Afiff 2023). *Vibrio* spp. that causes disease in shrimp, include *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio damsel*, *Vibrio campbellii*, *Vibrio splendidus*, and *Vibrio cholerae* (Aguilera-Rivera et al. 2019; Zou et al. 2020; Ulfiani et al. 2022).

As a consequence, disease control is a priority, especially in the context of supporting ecologically and economically sustainable aquaculture businesses. An integrated approach, such as prevention of infection through early detection of microbial species that can act as pathogens, boosting the host immune system with natural products or probiotics, controlling the spread of infection, and vaccine development and vaccination, is essential for the prevention and treatment of shrimp diseases (Cabello et al. 2016; Ina-Salwany 2019). However, prevention and treatment of vibriosis generally rely on antibiotics.

Antibiotics are used in modern aquaculture due to their effectiveness and the need for high yields (Miller and Harbottle 2018). In general, antibiotics have been applied to large quantities of shrimp feed and water, especially for treating and preventing diseases in shrimp culture (Zhou et al. 2018; Shao et al. 2021). As a result, antibiotic residues are present in aquaculture products and the environment, which serve as a lateral source of antibiotic resistant bacteria and antibiotic resistance genes among aquatic microorganisms (Chuah et al. 2016; Yuan et al. 2023). Other researchers reported that the uncontrolled and continued use of antibiotics in shrimp culture significantly contributes to the development of resistant *Vibrio* strains and reducing the effectiveness of antibiotic use (Rocha et

al. 2016; Sharma et al. 2021; Huang et al. 2022). Another effect to consider is the spread of resistance genes to other bacteria. Antimicrobial agents remain in sediment and aquatic environments, causing environmental degradation and conferring antimicrobial resistance to sediment bacteria (Yuan et al. 2023). A study examining 70 *Vibrio* strains from water and sediment samples in shrimp ponds in Brazil found resistance to several types of antibiotics, including ampicillin (AmP), ciprofloxacin (C), chloramphenicol (ClP), nitrofurantoin, gentamicin (CN), oxytetracycline, tetracycline (TE), and streptomycin (Rocha et al. 2016). Meanwhile, Yano et al. (2014) found *V. parahaemolyticus* in shrimp ponds in Thailand to be resistant to ampicillin and oxytetracycline (72% and 3%, respectively). Antibiotic resistance has also been reported in *V. cholera*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, and *V. campbellii*. The uninhibited application of antibiotics in shrimp culture leads to the formation of antibiotic sensitivity and resistance genes in bacteria.

Antibiotics and antibiotic-resistant bacteria are prevalent in aquaculture ecosystems. However, the impacts and interaction mechanisms of these substances in both biotic and abiotic media require further elucidation (Yuan et al. 2023). Considering the importance of research on the occurrence of antibiotic-resistant bacteria, understanding the diversity, content, and abundance of antibiotics in the environment can help researchers precisely control and remove antibiotics (Chen et al. 2022). To investigate the contamination status of antibiotics, this study aimed to determine the susceptibility pattern of pathogenic *Vibrio* bacteria and detect residual antibiotics in water and sediment in *P. vannamei* ponds. The results of this study may assist in policy-making to prevent disease outbreaks.

MATERIALS AND METHODS

Study area

The study was conducted in intensive ponds at the Marine Science Techno Park (MSTP), Universitas Diponegoro, Jepara, Central Java, Indonesia (Figure 1), in September 2022. This area was purposively selected and considered suitable as a leading point for designing outbreak prevention measures in intensive ponds at MSTP, Jepara. It had 8 square ponds, 18 circular ponds, and one semi-indoor pond. Currently, MSTP continues to develop hatchery and pond facilities to achieve a production capacity of 400 tons per year (DSTP UNDIP 2021). Based on Figure 1, sampling was conducted on 4 ponds, including a control pond with the closest location to the main water reservoir (not utilized for vaname shrimp culture activities). Pond 1 (vaname shrimp culture with a stocking density of 199 ind/m^2) was located closest to the control pond. Pond 2 (vaname shrimp farming with a stocking density of 199 ind/m^2) was located between pond 1 and pond 3. Pond 3 (vaname shrimp farming with a stocking density of 199 ind/m^2) was situated close to the final disposal pond destined for treatment before discharge into the sea. The total area of shrimp ponds is 314 m^2/unit, with a total stocking 278.964 shrimp fry/ponds.

Samples collection

Water and sediment samples from several *P. vannamei* ponds were collected using purposive sampling. Sampling was carried out in 3 areas (inlet, middle, outlet) of the ponds. Water samples were taken by hand at a depth of 50 cm and placed into 250 mL sample bottle then, labeled with the location. Sediment samples were collected using a sediment grab at the bottom of the pond and placed into sterile plastic bags similarly labeled. All the samples were placed in a cool box and transported to the laboratory for microbiological analysis. Care was taken to maintain sample integrity and avoid contamination during collection and transport.

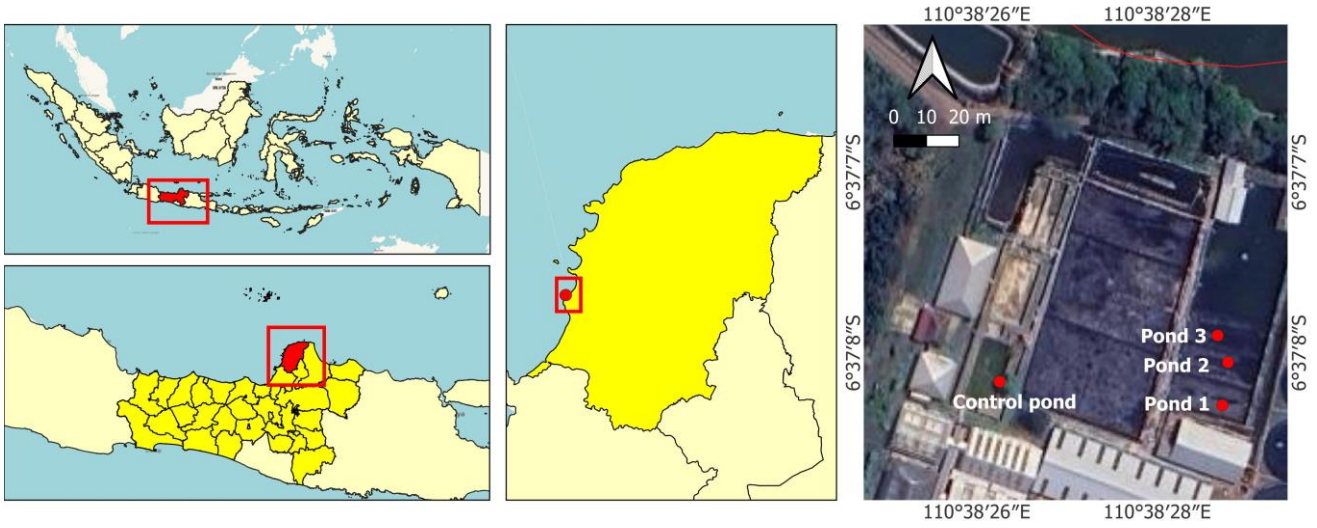

Figure 1. Geographical location of shrimp pond sampling sites at Marine Science Techno Park (MSTP), Universitas Diponegoro, Jepara, Central Java, Indonesia. Control pond (6°37'08.7"S 110°38'26.0" E), pond 1 (6°37'08.9"S 110°38'28.2" E), pond 2 (6°37'08.5"S 110°38'28.3"E), pond 3 (6°37'08.2"S 110°38'28.2"E)

Bacterial culture and isolation

Water samples were filtered through Thermo Scientific™ Nalgene™ Filter Unit equipped with nitrocellulose filter paper (Hawach Scientific). The filter paper has a pore size of 0.45 µm and a diameter of 47 mm. Both sediment and water samples were serially diluted (10^{-1} to 10^{-8}) in alkaline peptone water (HiMedia) and incubated for 24 hours.

To perform the dilution, samples were placed into a test tube containing 9 mL of sterile APW (10^{-1}) aseptically. The samples were then homogenized using a vortex. Subsequently, 1 mL of the 10^{-1} test tube was required before being transferred into the 10^{-2} test tube dilution. This process was repeated until the dilution reached a concentration of 10^{-8}. of each dilution, 100 µL was inoculated onto Thiosulfate Citrate Bile Salt Sucrose (TCBS; Difco) agar (Oxoid) supplemented with 2% NaCl and incubated at 37°C for 72 hours. Growing bacterial colonies were observed and characterized based on morphology, including shape, color, elevation, and edge (Cappuccino and Sherman 1996). Each colony with a different morphological appearance was purified using the streak plate method to obtain pure isolates (Sanders et al. 2012).

Total vibrio count

Total vibrio count (TVC) in water and sediment samples was determined using the serial dilution method with a range of dilutions from 10^{-1} to 10^{-8} in alkaline peptone water. The spread plate method was used in this experiment. TCBS agar plates were used to enumerate total vibrio bacteria. The plates were incubated in an inverted position at 37°C for 48 hours. Colonies were counted on plates containing 25-250 colonies using a colony counter. The total vibrio bacteria counts were presented as colony-forming units per mL (CFU/mL). Colony counts were calculated using the formula described by Buller (2014):

CFU/mL = N×dilution×plate dilution

Water quality

Water quality parameters, such as temperature (T), pH, salinity, and dissolved oxygen (DO) were measured directly during sampling. Water temperature was measured using an electronic thermometer with an accuracy of 0.1°C. pH was measured using a HANNA® HI98129 pH meter with an accuracy 0.01. Salinity was measured using an ATAGO® PAL-06s refractometer with an accuracy of 1 ppt. DO was recorded using a YSI@Pro DO meter (read to 0.1 mg L^{-1}). Organic waste was quantified by measuring the concentrations of Total Ammonia Nitrogen (TAN), Nitrogen dioxide (NO_2), and Nitrate ($NO_{3)}$ using spectrophotometric methods at 630 nm (Isnansetyo et al. 2014).

Antibiotic resistance

Antimicrobial susceptibility test was performed using the Kirby-Bauer disc diffusion method (Bauer et al. 1966) according to Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI 2015; Baron et al. 2020). Six types of commercial antibiotic discs (OXOID) were used: Gentamicin (CN, 10 µg), Ampicillin (AMP, 10 µg), Ciprofloxacin (CIP, 5 µg), Tetracycline (TE, 30 µg), Chloramphenicol (C, 30 µg), and Amoxicillin (AML, 25 µg).

The bacteria were cultured in Nutrient Broth (NB) at 37°C for 24 h and diluted using 2% NaCl solution until the suspension became slightly turbid. The suspension was then adjusted to 0.5 McFarland standard (3×10^8 CFU mL^{-1}) (Uddin et al. 2018). *Vibrio* spp. isolates grown in liquid media were swabbed on Mueller-Hinton media in a Petri dish. Antibiotic discs were then placed on top of the media containing *Vibrio* bacterial culture. All plates were incubated at 37°C for 24 h. The diameter of inhibition zone was measured with digital calipers. Interpretive criteria for disc diffusion susceptibility testing of *Vibrio* spp. are shown in Table 1. The performance of the zone of inhibition was categorized as resistant, intermediate, or sensitive based on the Clinical and Laboratory Standards Institute M45 guidelines (CLSI 2015).

Molecular characterization by 16S rRNA gene sequencing

The genomic DNA of *Vibrio* isolates was extracted using the Chelex 100 DNA extraction method (Yang et al. 2024). The DNA extraction consisted of lysis, binding, washing, and elution steps. PCR amplification was performed using MyTaq HS Red Mix (Bioline, BIO-25047).

The DNA was amplified for the 16S rRNA gene using polymerase chain reaction (PCR) using the following universal primer: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the following specific eubacterial primer: 1492R (5'-TACGGTTAACCTTGTTACGACTT-3').

Table 1. Interpretive criteria for disc diffusion susceptibility test of *Vibrio* spp.

Antibiotics	Zone diameter (mm) interpretive criteria		
	Resistance	Intermediate	Sensitive
Gentamicin (CN)	≤12	13-14	≥15
Ampicillin (AmP)	≤13	14-16	≥17
Ciprofloxacin HCL (C)	≤15	16-20	≥21
Tetracycline (TE)	≤11	12-14	≥15
Chloramphenicol (ClP)	≤12	13-17	≥18
Amoxicillin (Aml)	≤13	14-17	≥18

The 25 µL PCR reaction consisted of 2.0 µL of each primer, 8.5 µL ddH2O, and 12.5 µL Go Taq Polymerase. The 16S rRNA gene was amplified with an MJ Mini Personal Thermal Cycler (BIO-RAD). PCR conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. Samples were then stored at 4°C (Amalia et al. 2021). PCR products were visualized using 1% agarose gel electrophoresis, and the results were observed with UVIDoc HD5 (UVITEC Cambridge). All PCR products estimated at ~1,500 bp were sequenced at 1st Base Malaysia for further analysis.

The sequencing results were compared to other sequences from the GenBank database at the National Center for Biotechnology Information (NCBI), National Institute for Health (NIH), USA (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1997). Phylogenetic analysis of active bacterial isolates was performed by aligning sequences using CLUSTAL-W (MEGA X software). The aligned data set was used for phylogenetic analysis using the neighbor-joining tree method with the Kimura 2-parameter model and 1,000 bootstrap replications (Kumar et al. 2018). The corresponding sequence of *Aspergillus niger* was used as an outgroup for rooting the tree.

RESULTS AND DISCUSSION

Shrimp culture is one of the most essential aquaculture commodities contributing to the global economy. *P. vannamei* is regarded as the most important cultivated species in the world, with 5 million metric tons produced annually (FAO 2016). The outbreak of bacterial diseases, especially those caused by *Vibrio*, has become an important factor restricting the stable production of shrimp culture. *Vibrio* spp. are significant pathogens in many aquaculture systems and are abundant in tropical and temperate marine environments (Ina-Salwany et al. 2019). Moreover, it can cause up to 100% mortalities in aquatic animals (Kumar et al. 2014).

Diversity of *Vibrio* spp.

In the present study, a total of 33 water and sediment samples collected from MSTP ponds were analyzed. The abundance of *Vibrio* sp. (TVC) in different shrimp pond samples is presented in Figure 2. The TVC ranged from 0.46×10^7 CFU mL^{-1} to 7.07×10^7 CFU mL^{-1} in water samples and from 4.73×10^7 CFU mL^{-1} to 20.07×10^7 (2.01×10^8) CFU mL^{-1} in sediment samples. Based on the data, the abundance of *Vibrio* sp. in sediment was higher than in water for all ponds. The highest total vibrio count was found in pond 1 sediment samples (2.01×10^8 CFU mL^{-1}), while the lowest was found in the water samples from the control pond (0.46×10^7 CFU mL^{-1}). This aligns with previous studies by Sampaio et al. (2022), who found that all *Vibrio* species are aquatic heterotrophs living between the sediments and the water column. Rao and Surendran (2013) found that in aquaculture, total vibrio counts in pond sediment samples (2.2×10^3 CFU g^{-1}) were higher than in pond water samples (2.0×10^2 CFU mL^{-1}). Moreover, Li et al. (2002) reported that the number of *Vibrios* in pond sediment was 10 to 20 times higher than in the water column. Furthermore, in another study the five most abundant phyla of bacteria present in the sediment of the fish pond over a three-year period, including Proteobacteria, Chloroflexi, Firmicutes, Bacteroidetes, and Planctomycetes (Feng et al. 2022). The higher bacterial abundance in pond sediments was attributed to organic matter accumulation at the pond bottom. This is consistent with research by Rao and Surendran (2013), Diner et al. (2021), and Aisyah (2022), who found that pathogenic bacteria such as *Vibrio* sp. increase as organic load and nutrient levels in ponds increase.

Nevertheless, results of present study revealed that the abundance of *Vibrio* sp. in both water and pond sediment was much higher (0.46×10^7 to 2.01×10^8 CFU mL^{-1}) compared to other previous studies. *V. harveyi* was detected in postlarvae, and water was 150-1.1×10^8 g^{-1} postlarvae and 7-4.6×10^4 CFU mL^{-1} of water samples, respectively (Thaithongnum et al. 2006). According to Regulation by the Minister of Marine Affairs and Fisheries of the Republic of Indonesia No. 75 of 2016, the maximum limit of aquatic *Vibrio* is 10^3 CFU mL^{-1}. Raungpan et al. (1995) also found that when *Vibrio* and luminous bacteria exceeded 10^4 cells mL^{-1} in overcrowded cultured shrimp ponds, it caused severe health problems for the shrimp. Supono et al. (2019) reported that in vannamei shrimp, *Vibrio* sp. abundance exceeding 10^5 CFU mL^{-1} was susceptible to attack by white feces disease (WFD). *Vibrio* bacteria sensitive to environmental conditions can become opportunistic if their abundance exceeds the threshold (Heenatigala and Fernando 2016). This can lead to mass mortality caused by physiological symptoms resulting from *Vibrio* bacterial infections in shrimp culture (Sriurairatana et al. 2014; Mastan 2015; Aranguren et al. 2017).

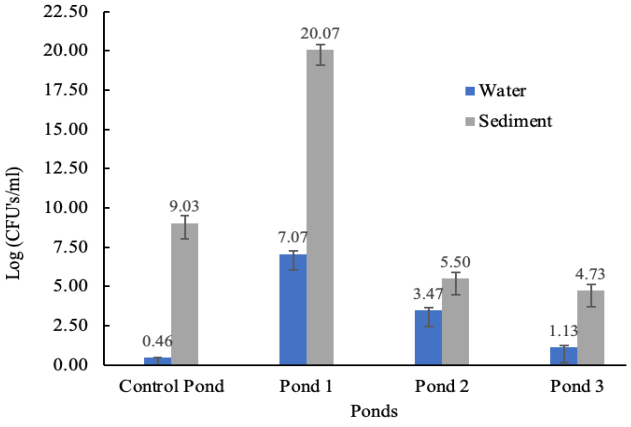

Figure 2. Total vibrio count (TVC) in water and sediment samples from different shrimp ponds

In this study, 150 *Vibrio* sp. were isolated from water and sediment shrimp pond samples. Yellow, greenish-yellow, green, and dark green bacterial colonies appeared on TCBS agar (Figure 3). However, yellow colonies were the dominant bacteria compared to other colors. According to Supono et al. (2019), the yellow color of the colonies is thought to be due to their ability to ferment sucrose and reduce pH on TCBS media, while the green color of *Vibrio* colonies is due to the bacteria's inability to ferment sucrose. Additionally, a higher presence of green colony *Vibrio* is associated with higher virulence and is widely claimed to be the cause of disease for shrimp in ponds. Meanwhile, dark green colonies are suspected to be caused by the growth of *Photobacterium damselae* (Hassanzadeh et al. 2015). Furthermore, 15 of the 150 bacterial isolates were selected to characterize the morphology of colony (Table 2).

Water quality

Water quality conditions and nutrient availability are limiting factors for the growth of microorganisms in the pond ecosystem. Therefore, microbiological parameters were used as water quality indicators for groups of pathogenic bacteria, the most common cause of disease in shrimp culture, especially *Vibrio* sp. Furthermore, nutrient availability influences the population of *Vibrio* sp. and is closely related to its growth ability (Smith et al. 2018; Wu et al. 2018).

Temperature, DO, pH, salinity and nitrite parameters of this research were within acceptable limits. However, nitrate and TAN (Total Ammonia Nitrogen) levels exceeded the recommended ranges (Table 3). The temperature ranged from 28.0°C to 28.5°C, DO levels ranged from 6.81 to 7.93 mg/L, pH from 8.12 to 8.25, and salinity was 31 ppt in all ponds. Nitrite ranged from 0.013 to 0.085 mg/L, nitrate from 0.5 to 1.36 mg/L, and TAN from 0.02 to 0.89 mg/L. In control ponds, the parameters of nitrate and TAN were within recommended ranges. However, these two parameters exceeded the recommended ranges in ponds 1, 2, and 3.

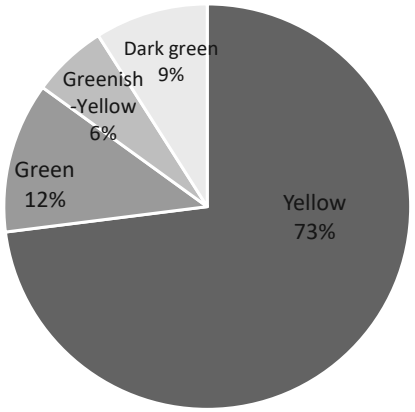

Table 2. Morphological characteristics of bacteria colonies isolated from *Penaeus* vannamei ponds

Table 2. Morphological characteristics of bacteria colonies isolated from *Penaeus vannamei* ponds

Samples code	Macroscopic characteristics				
	Colony size	Shape	Margin	Texture	Color
T3.S.1	Small	Round	Entire	Smooth	Yellow
T3.A.O	Large	Round	Entire	Smooth	Yellow
T2.S1	Moderate	Round	Entire	Smooth	Green
T1.A.O	Moderate	Round	Entire	Smooth	Yellow
T2.A.TG	Small	Round	Entire	Smooth	Yellow
TK.A.I	Punctiform	Round	Entire	Smooth	Yellow
T1.A.I	Small	Round	Entire	Smooth	Yellow
T1.A.TG	Small	Round	Entire	Smooth	Yellow
T3.B.1	Punctiform	Round	Entire	Smooth	Dark Green
T1.B1	Punctiform	Round	Entire	Smooth	Dark Green
T2.B	Small	Round	Entire	Smooth	Yellow
T2.A.O	Punctiform	Round	Entire	Smooth	Yellow
T1.S1	Small	Round	Entire	Smooth	Yellow
T3.A.TG	Small	Irregular	Undulate	Smooth	Green
TK.A.O	Small	Irregular	Undulate	Smooth	Green

Table 3. Water quality parameters in *Penaeus vannamei* ponds

Samples	Variables						
	Temp. (°C)	DO (mg/L)	pH	Salinity (ppt)	Nitrite (mg/L)	Nitrate (mg/L)	TAN (mg/L)
Control pond	28.0	7.93	8.12	31	0.013	0.5	0.02
Pond 1	28.1	7.54	8.25	31	0.085	1.35	0.85
Pond 2	28.5	6.81	8,25	31	0.027	1.36	0.89
Pond 3	28.1	7.39	8.20	31	0.011	0.78	0.15
Recommended ranges	28-30[a]	>4[b]	7.5-8.5[b]	26-32[a]	<0.5[c]	0.4-0.8[d]	< 0.1[a]

Notes: a: PERMEN KP (2016); b: Venkateswarlu et al. (2019); c: Nkuba et al. (2021); d: Torun et al. (2020)

Ammonia is toxic to shrimp at high concentrations; the maximum limit of ammonia concentration in waters is <0.1 mg/L (PERMEN KP 2016). The high concentration of TAN in the present study (Table 3) was suspected to be caused by the accumulation of organic materials such as high stocking density, leftover feed with high protein content, feces, and waste from shrimp metabolism. Uneaten feed and feces are decomposed by bacteria, during which organic carbon is oxidized to carbon dioxide, and organic nitrogen is mineralized to ammonia, nitrite, and nitrate (Boyd et al. 2020).

An increase in organic material encourages the microflora to develop into opportunistic pathogens, including populations of *Vibrio* spp. (Heenatigala and Fernando 2016). *Vibrio* sp. requires organic material as a carbon source to synthesize proteins and for growth (Smith et al. 2018; Wu et al. 2018). According to Chatterjee et al. (2014), *Vibrio* utilizes ammonia as a nitrogen source for energy and other organic compounds for forming protoplasmic cells, especially in forming cell walls. Likewise, Vibrios are also agents of organic matter mineralization, playing an essential role in organic matter recycling due to their enzymes, which allow them to use a wide variety of substrates (Thompson and Polz 2006).

The nitrate concentration in the ponds was high at 0.5-1.36 mg/L, exceeding the standard limit for total nitrate in shrimp ponds. The presence of nitrate in shrimp pond waters is due to nitrite oxidation by *Nitrobacter* bacteria. Therefore, the amounts of nitrite and ammonia can be used to determine the nitrate levels. In addition, nitrate is a vital N component that *Vibrio* uses as a nutrient acceptor (Wang et al. 2016). However, increasing nitrate (NO_3^-) is thought to cause algal overgrowth (Harke et al. 2016). Furthermore, the elevated nitrogen levels in the water and sediment are believed to be a consequence of the accumulation of antibiotics, disrupting the nitrogen-cycling microorganisms within aquaculture systems (Li et al. 2024).

Molecular characterization

A total of 4 isolates from 15 bacteria colonies were selected based on morphological differences and different phenotypic identifications, as representative strains of all isolates to be subjected to detailed molecular characterization using 16S rRNA. All four isolates produced PCR products of approximately 1500 bp in length (Figure 4). Sequencing analysis revealed that T3.S.1, T1.B.1, T2.S.1, and TK.A.1 had lengths of 1425 bp, 1351 bp, 1424 bp, and 1418 bp, respectively (Table 4).

After sequencing, PCR amplification results of the 16S rRNA gene were edited to remove ambiguous nucleotides. The consensus DNA sequences were then compared to the BLAST database on NCBI. Advanced analysis through sequencing showed that T3.S.1, T1.B.1, T2.S.1, and TK.A.1 belonged to the genus *Vibrio* sp., with closest relativity to *Vibrio alginolyticus* strain BG17, *P. damselae* strain TZH7, *Vibrio parahaemolyticus* strain VP9, and *Vibrio harveyi* strain S09080, respectively. All isolates showed 100% homology with their closest relatives (Table 4).

A total of 15 DNA sequences were aligned to create the radial phylogenetic tree. The isolates with the highest homology were consistently found in the same clade (Figure 5). The T3.S.1 is similar to *Vibrio alginolyticus* strain BG17, isolated from a shrimp pond (Alagappan et al. 2010). *V. alginolyticus* is the most dominant pathogenic *Vibrio* species causing vibriosis in hatchery water, post-larvae samples, shrimp pond water, sediment, and shrimp larvae (Rao and Surendran 2013). It has also been reported as a pathogen in *Penaeus monodon* Fabricius, 1798, and produces mortality rates of up to 81-86% in juvenile shrimp (Abdul Hannan et al. 2019).

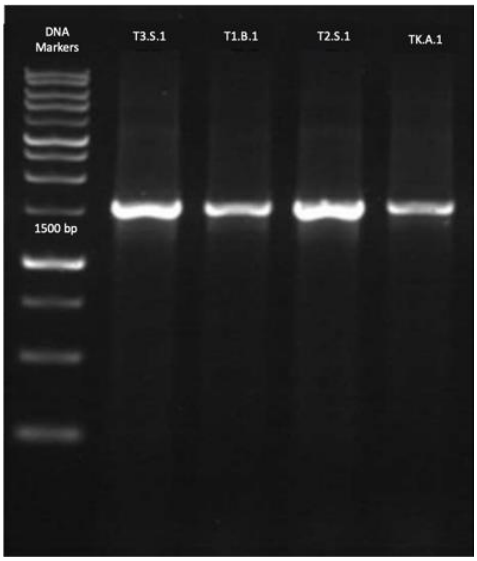

Figure 4. Gel electrophoresis of 16S rRNA gene amplification products from selected *Vibrio* isolates

Table 4. BLAST homology results of *Vibrio* spp. isolated from *Penaeus vannamei* ponds

Isolates code	Length (bp)	Closest relativity	Homology	Reference accession number
T3.S.1	1327	*Vibrio alginolyticus* strain BG17	100%	HQ694832.1
T1.B.1	1396	*Photobacterium damselae* strain TZH7	100%	MT071412.1
T2.S.1	1424	*Vibrio parahaemolyticus* strain VP9	100%	MF372385.1
TK.A.1	1418	*Vibrio harveyi* strain S090801	100%	HM236045.1

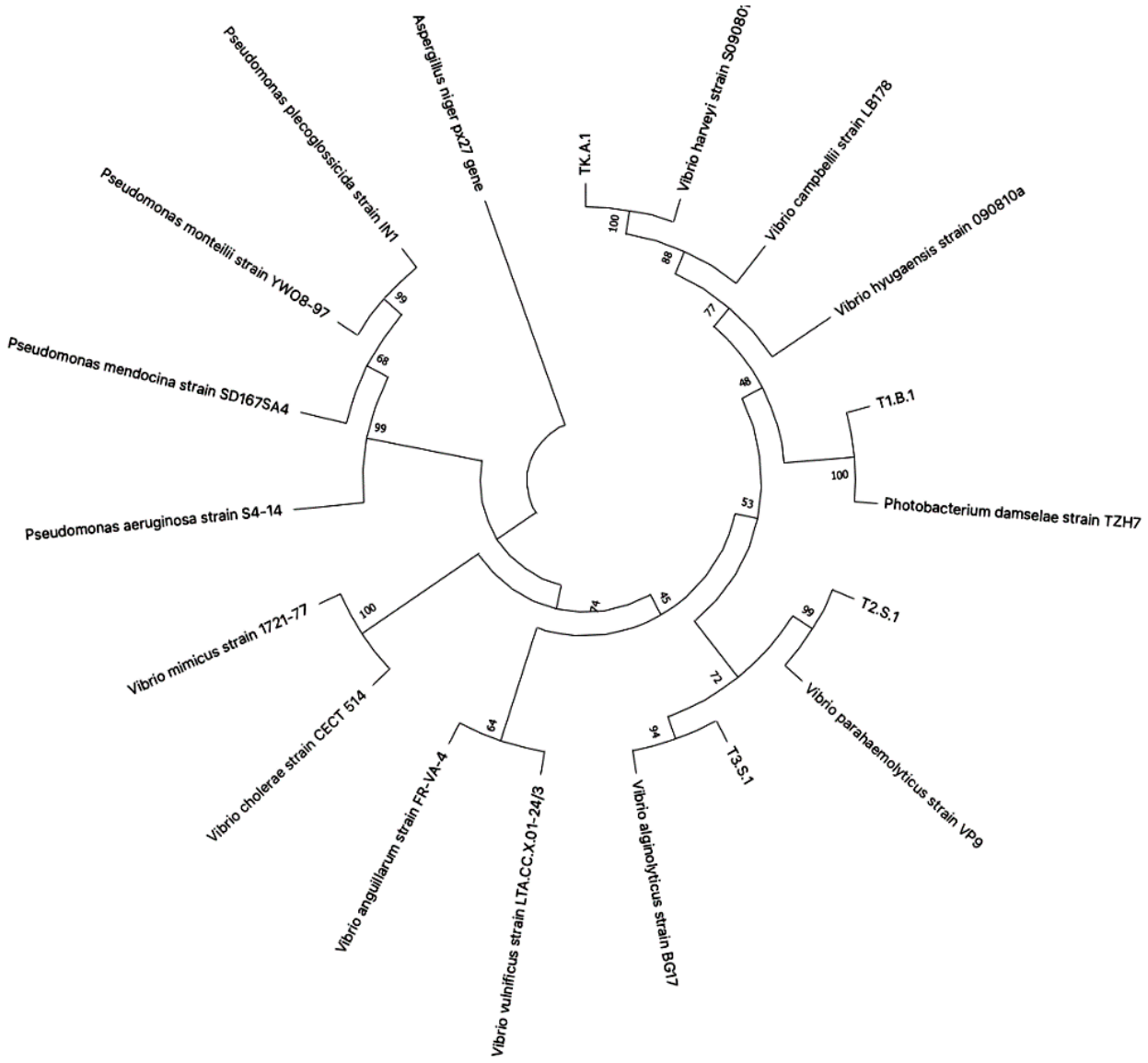

Figure 5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of *Vibrio* spp. and isolated from *Penaeus vannamei* ponds, with the neighbor-joining approach and 1000 bootstrap replications. *Aspergillus niger* was used as an outgroup

The T1.B.1 is similar to the *P. damselae* strain TZH7, found in mariculture (Gouife et al. 2022). According to Vaseeharan et al. (2007), these bacteria are normal inhabitants of seawater and marine sediments, preferring warm water conditions (20-30°C). *P. damselae* is pathogenic to a wide range of aquatic animals such as fish, crustaceans, mollusks, and cetaceans. Additionally, *Photobacterium damselae* ssp. *damselae* are also pathogenic to a few shrimp species, including *P. monodon*, *Exopalaemon carinicauda* Holthuis 1950, and *Litopenaeus vannamei* Boone 1931 (Vaseeharan et al. 2007; Liu et al. 2016; Aguilera-Rivera et al. 2019). Wang et al. (2020) reported that *P. damselae* subsp. *damselae* isolated from hepatopancreas and gills of diseased shrimp, caused massive mortalities (over 80%) in cultured *Penaeus vannamei* in Hainan Province, China. Singaravel et al. (2020) also reported that *P. damselae* could cause up to 100% mortality in *L. vannamei*. Petchimuthu et al. (2022), revealed that cobia and silver pompano showed 100% and 85% mortality at a bacterial concentration of 3.5×10^4 CFU Fish^{-1} and 2.75×10^6 CFU Fish^{-1} respectively.

Isolate T2.S.1 showed similarities with *V. parahaemolyticus* strain VP9, isolated from the bottom soil of a shrimp pond in Umas Sabah, Malaysia, after AHPND outbreaks (Lee et al. 2017). *V. parahaemolyticus* has been found in sediment, water, and shrimp farm samples (Silvester et al. 2015). Recently, it has been increasingly reported to cause disease in *P. vannamei* (Zhang et al. 2021), with acute hepatopancreatic necrosis disease (AHPND) being the most severe disease currently affecting shrimp farms (Raja et al. 2017). *V. parahaemolyticus* is also the cause of glass post-larvae disease, also known as highly lethal *Vibrio* disease (HLVD), which poses a severe threat to shrimp farming (Yang et al. 2022). Mass mortality can exceed 70% (Kongrueng et al. 2015), although different strains of *V. parahaemolyticus* have varying levels of virulence. Some less virulent strains do not cause 100% mortality (Soto-Rodriguez et al. 2015).

TK.A.1 is most closely related to *Vibrio harveyi* strain S090801, a severe bacterial pathogen of marine fish and invertebrates, including penaeid shrimp, in aquaculture (Zhang et al. 2020). *V. harveyi* in shrimp farming systems is found to be associated with hatcheries, where it can be isolated from incoming seawater, broodstock, larvae, juveniles, and rearing tank water (Zhang et al. 2020; Peeralil et al. 2020; Li et al. 2024). In India, luminescent *V. harveyi* has been reported to cause 100% mortality in penaeid shrimp larvae at the nauplii, mysis, and postlarva stages (Kumar et al. 2021). Furthermore, *V. harveyi* has been identified in China as the etiologic pathogen of bacterial white tail disease (BWTD) and exhibits high virulence (Zhou et al. 2012).

Antibiotic resistance

Antibiotics are commonly used in aquaculture to treat bacterial infections, and most *Vibrio* spp. are typically susceptible to them (Kah Sem et al. 2023). However, after only a few years of use, pathogens have acquired resistance to various antibiotics (Andreoni and Magnani 2014). This resistance has developed due to the widespread and frequent use of antibiotics in aquaculture (Kah Sem et al. 2023). The emergence of resistant bacteria and resistance genes can impact treatment effectiveness and public health by spreading antibiotic-resistant bacteria to consumers (Uddin et al. 2021).

Four isolates of *Vibrio* spp., designated as T3.S.1, T1.B.1, T2.S.1, and TK.A.1, were selected for antibiotic resistance test. These isolates were obtained from water and sediment samples. Six antibiotics (gentamicin, ampicillin, chloramphenicol, ciprofloxacin, tetracycline, and amoxicillin) recommended by the Clinical and Laboratory Standards Institute (CLSI) for the treatment of *Vibrio* spp. were used in the test. Based on the results, all antibiotics showed some level of antibacterial activity. Almost all isolates were resistant to gentamicin, ampicillin, ciprofloxacin, tetracycline, and amoxicillin, while *P. damselae* showed intermediate resistance to chloramphenicol (Table 5). Previous studies using the similar antibiotics also showed resistance to *Vibrio* sp. (Shaw et al. 2014; Yudiati et al. 2021).

Table 5 shows that *P. damselae* exhibited the largest inhibition zone (17.5±2.12 mm) against chloramphenicol. The lowest inhibition zones were observed for *V. alginolyticus* and *V. harveyi* (0.5±0.70 mm) against amoxicillin and tetracycline, respectively. *V. parahaemolyticus* showed no antimicrobial activity for any tested antibiotics, indicating complete resistance.

Vibrio alginolyticus showed resistance to all six tested antibiotics (Table 6). Similar studies have found *V. alginolyticus* to be resistant to ampicillin, gentamicin, and tetracycline in aquatic environments in coastal mariculture areas in China (Yu et al. 2022). Isnansetyo et al. (2022) reported that *V. alginolyticus* isolated from the Spermonde Islands, Indonesia, was resistant to oxytetracycline, ampicillin, erythromycin, kanamycin, and enrofloxacin. However, Damir et al. (2013) found *V. alginolyticus* resistant to ampicillin but immediately resistant to tetracycline and sensitive to chloramphenicol. Abdel-Aziz et al. (2013) reported that *V. alginolyticus* was sensitive to ciprofloxacin, gentamicin, enrofloxacin, and tetracycline but resistant to ampicillin.

Photobacterium damselae exhibited resistance to gentamicin, ampicillin, ciprofloxacin, tetracycline, and amoxicillin but showed intermediate resistance to chloramphenicol. These results align with Wang et al. (2020), who found that *P. damselae* subsp. *damselae* from *P. vannamei* white shrimp was resistant to tetracycline and gentamicin but sensitive to chloramphenicol. *P. damselae* in fish reared in Greek aquaculture facilities was sensitive to ampicillin, gentamycin, tetracycline, ciprofloxacin, and amoxcicilin Chiu et al. (2013), observed resistance to chloramphenicol, gentamicin, amoxicillin, penicillin, enrofl, and lumequine (Lattos et al. 2022), oxacin, sulfamethoxazole/ trimethoprim, ampicillin, oxolinic acid, florfenicol, and tetracycline in 50 isolates of *P. damselae* derived from mackerel and cobia. In another study, around 90% of the *P. damselae* subsp. *damselae* isolates were found to be multiple antibiotic-resistant and carried antibiotic resistance genes such as beta-lactam and streptomycin genes from the fish of the southeast coast of India (Petchimuthu et al. 2022).

Table 5. Antibiotic inhibition zone diameters (mm) for selected *Vibrio* isolates

Bacterial isolates	Cn* (10 µg)	Amp* (10 µg)	Cip* (5 µg)	Te* (30 µg)	C* (30 µg)	Aml* (25 µg)
V. alginolyticus	1.50±0.71	1.50±0.71	9.00±1.41	0.00±0.00	1.50±0.71	0.50±0.71
P. damselae	7.00±0.00	6.50±2.12	13.50±2.12	8.00±1.41	17.50±2.12	2.00±1.41
V. parahaemolyticus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
V. harveyi	2.00±0.00	1.00±0.00	6.00±2.83	0.50±0.71	2.00±0.00	1.00±0.00

Notes: Gentamicin (Cn); Ampicilin (Amp); Ciprofloxacin (Cip); Tetracycline (Te); Chloramphenicol (C); Amoxcicilin (Aml)

Table 6. Antibiotic susceptibility profiles of selected *Vibrio* isolates

Bacterial isolates	Cn* (10 µg)	Amp (10 µg)	Cip (5 µg)	Te (30 µg)	C (30 µg)	Aml (25 µg)
V. alginolyticus	Resistance	Resistance	Resistance	Resistance	Resistance	Resistance
P. damselae	Resistance	Resistance	Resistance	Resistance	Intermediate	Resistance
V. parahaemolyticus	Resistance	Resistance	Resistance	Resistance	Resistance	Resistance
V. harveyi	Resistance	Resistance	Resistance	Resistance	Resistance	Resistance

Notes: Gentamicin (Cn); Ampicilin (Amp); Ciprofloxacin (Cip); Tetracycline (Te); Chloramphenicol (C); Amoxcicilin (Aml)

Furthermore, *V. parahaemolyticus* displayed resistance to gentamicin, ampicillin, ciprofloxacin, tetracycline, chloramphenicol, and amoxicillin. These results align with the findings of Letchumanan et al. (2015), who demonstrated that *V. parahaemolyticus* isolates from shrimp samples exhibited high susceptibility to ampicillin-sulbactam (96%), chloramphenicol (95%), imipenem (98%), tetracycline (82%), and trimethoprim-sulfamethoxazole (93%). Lopatek et al. (2015) also reported that all isolates of *V. parahaemolyticus* from raw shellfish were susceptible to chloramphenicol and tetracycline. Similarly, Xu et al. (2016) demonstrated that the majority of *V. parahaemolyticus* isolates from retail aquatic products in North China were susceptible to chloramphenicol (95%), ciprofloxacin (92%), gentamicin (63%), tetracycline (83%), and trimethoprim-sulfamethoxazole (75%). Finally, *V. parahaemolyticus* isolates from various seafood types in Selangor, Malaysia showed high resistance to ampicillin, cefazolin, and penicillin, intermediate resistance to cefotaxime and ciprofloxacin, and sensitivity to chloramphenicol, amoxicillin-clavulanic acid, doxycycline, imipenem, meropenem, tetracycline, and gentamicin (Tan et al. 2020).

Vibrio harveyi resisted gentamicin, ampicillin, ciprofloxacin, tetracycline, chloramphenicol, and amoxicillin. This resistance pattern is comparable to previous findings on *V. harveyi* from shrimp ponds on the southeast coast of India, where the bacteria were resistant to ampicillin, cefaclor, ciprofloxacin, penicillin, rifampicin, vancomycin but sensitive to gentamicin, norfloxacin, and chloramphenicol (Stalin and Srinivasan 2016). Nurhafizah et al. (2021) also reported *V. harveyi* isolates in pacific white shrimp from Malaysia were resistant to oxytetracycline, oleandomycin, amoxicillin, ampicillin, and colistin but were sensitive to oxolinic acid. Another study found *V. harveyi* resistant to vancomycin, amoxicillin, midecamycin, furazolidone, tobramycin, rifampicin, gentamicin, and tetracycline but sensitive to erythromycin, trimethoprim-sulfamethoxazole, doxycycline, chloramphenicol, florfenicol, norfloxacin, and ciprofloxacin (Deng et al. 2020).

The results of present findings revealed the presence of *Vibrio* spp. at high-density levels (10^7-10^8 CFU mL^{-1}) from water and sediment samples at MSTP Jepara shrimp pond, Indonesia. This is the first report of *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, and *P. damselae* showing resistance to gentamicin, ampicillin, ciprofloxacin, amoxicillin, tetracycline, chloramphenicol, and at this location. *P. damselae* showed intermediate resistance to chloramphenicol.

These findings highlight the importance of planning measures to prevent vibriosis outbreaks. Good aquaculture practices, improved health management plans, treatment using alternative bacterial control methods, and monitoring of drug usage in aquaculture should be encouraged to reduce the risk of emerging antibiotic-resistant bacteria in shrimp aquaculture. These measures could evolve into effective therapies to benefit aquatic livestock.

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