Diversity of Antibiotic-Resistant Bacteria Identified from Intensive Shrimp Farming Areas in the Coastal Area of Kendal Regency

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Abstract

Intensive *Pennaeus vannamei* aquaculture is a critical reservoir for the emergence of antimicrobial resistance (AMR), driven by the extensive use of antibiotics under high-density production conditions. This study aimed to isolate, characterize, and identify antibiotic-resistant bacteria from intensive shrimp pond environments in the Rowosari and Kaliwungu Districts of Kendal Regency, Central Java, Indonesia. Water, sediment, and shrimp samples were collected via purposive sampling and cultured on thiosulfate-citrate-bile salts sucrose (TCBS) and Zobell Marine Agar. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method, following CLSI guidelines, against erythromycin (ERY), oxytetracycline (OXY), enrofloxacin (ENR), and amoxicillin (AMX). Molecular identification was conducted via 16S rRNA gene sequencing with BLAST homology analysis and neighbor-joining phylogenetic reconstruction using MEGA-X. Four isolates were recovered and characterized in this study. Oxytetracycline resistance was detected universally, and isolate A23 exhibited complete resistance to all tested antibiotics. Molecular analysis identified A23 as *V. injensis* strain VS1, B15 as *V. metschnikovii* strain Vm-4, and B12 and B17 as *Staphylococcus arlettae*, each with 100% sequence homology with reference strains. These findings confirm the presence of diverse AMR bacterial assemblages in intensive shrimp pond environments in Kendal Regency and underscore the critical need for integrated AMR surveillance and prudent antibiotic stewardship in Indonesian shrimp aquaculture.

Keywords: Antibiotics, Antibiotic-resistant bacteria, *P. vannamei*, *S. arlettae*, *Vibrio* sp.

INTRODUCTION

Penaeus vannamei is a brackish aquaculture commodity that holds crucial control for ensuring food security because of its high demand and global production. In 2020, the total production of *P. vannamei* reached 5.8 million tons and is expected to continue to increase (FAO, 2022). Data show a 10% annual increase in shrimp demand over the past three decades (Prabina *et al*., 2023). This increase in demand was followed by an increase in the area of farmland. In Indonesia, Kendal Regency is one of the largest vanamei shrimp-producing areas in Central Java. Various intensifications have been carried out to meet this demand, including the cultivation system and conversion of agricultural land into ponds (Asmild *et al*., 2023; Setyawan *et al*., 2022). Due to the culture species variant include *P. monodon* and *P. vannamae*, and variant of aquaculture technology applied their production in this area has still steadily increased (Sarjito and Sabdono, 2021). Despite rapid aquaculture intensification, limited information exists about the occurrence and pathogenic characteristics of these bacteria. Previous studies focused on antimicrobial residues or general resistance in aquaculture systems, while comprehensive characterization of antibiotic-resistant bacteria from intensive P. vannamei ponds is scarce. This intensification is accompanied by increased inputs to the environment through feeding, medicine, biosecurity, etc (Baki *et al*., 2023). The use of drugs, including antimicrobials, in cultivation activities can cause side effects that indirectly endanger the safety of consumers. One of the threats of bacterial resistance stems from the continuous use of antimicrobials in the fisheries sector.

Global antimicrobial consumption in aquaculture in 2017 was estimated at 10,259 tons, with 93% of the total in the Asia-Pacific region. Indonesia ranks third for the highest antimicrobial consumption in Asia-Pacific at 9.8% (Schar *et al*., 2020). This amount is approximately 935 tons and is

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still estimated to increase by 55% by 2030. Repeated and unregulated antimicrobial exposure in aquaculture environments selects for bacteria that exhibit resistance to one or multiple drug classes, a phenomenon known as antimicrobial resistance (AMR). Resistance to antibiotics frequently applied in shrimp aquaculture, such as erythromycin (ERY), oxytetracycline (OXY), enrofloxacin (ENR), and amoxicillin (AMX), has been reported in bacteria isolated from shrimp ponds and pond sediments, which represent four distinct antimicrobial classes — macrolides, tetracyclines, fluoroquinolones, and β-lactams, respectively — and are among the most commonly used antimicrobials in shrimp farming operations globally, including in the Southeast Asian region (Prabina *et al*., 2023; Hirshfeld *et al*., 2023). In particular, resistance to oxytetracycline is commonly detected in aquaculture-associated bacteria and may reduce the effectiveness of disease treatment in cultured shrimp (Zhang *et al*., 2024). The presence of antibiotic-resistant bacteria in pond environments also indicates that intensive aquaculture systems may serve as sources for the persistence and spread of resistant bacteria in aquatic ecosystems (Iftehimul *et al*., 2025; Rodríguez-Blanco *et al*., 2025). Previous studies further reported that several Vibrio species isolated from shrimp farming areas showed resistance to tetracycline, macrolide, fluoroquinolone, and β-lactam antibiotics, highlighting potential risks for aquaculture production and environmental health (Devadas *et al*., 2025; Ortega *et al*., 2025).

Previous investigations have documented AMR in bacteria isolated from shrimp aquaculture systems across Southeast Asia and Indonesia; the majority of these studies have been conducted in geographically distinct or ecologically dissimilar settings (Prabina *et al*., 2023; Devadas *et al*., 2025; Ortega *et al*., 2025). Furthermore, although several *Vibrio* species isolated from shrimp farming areas have been shown to exhibit resistance to multiple antibiotic classes, the concurrent occurrence of non-*Vibrio* resistant genera in intensive shrimp pond environments has received comparatively little attention. The present study, therefore, addresses this gap by isolating, identifying, and characterizing bacteria from intensive vannamei shrimp ponds in Kendal Regency, evaluating their antimicrobial resistance profiles, and contributing locality-specific data toward evidence-based AMR management in Indonesian shrimp aquaculture. From this research, we also wanted to discover what AMR bacterial species are present in intensive vannamei shrimp ponds in Kendal Regency, Central Java, and which of these exhibit antimicrobial-resistant phenotypes as defined by resistance to at least one antimicrobial agent in three or more antibiotic classes.

MATERIALS AND METHODS

This study was conducted in intensive ponds in the Rowosari and Kaliwungu Districts in Kendal Regency, Central Java. The sampling location was at two different points, with three pond maps from each site (Figure 1).

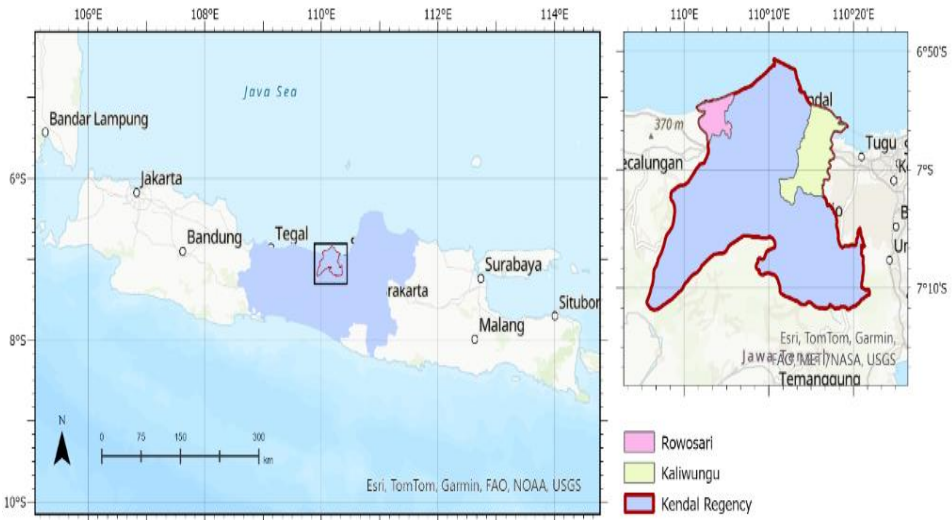

Figure 1. Study area that located in Rowosari and Kaliwungu, Kendal regency

Table 1. The interpretation level of antibiotic resistance based on the CLSI

Antibiotik	Antimicrobial resistance diameter (mm)		
	Sensitive (S)	Mediate sensitive (MS)	Resistant (R)
ERY (15 µg)	≥ 23	14-22	≤13
OXY (30 µg)	≥ 19	15-18	≤14
ENR (5 µg)	≥ 23	14-22	≤13
AMX (20 µg)	≥ 18	14-17	≤13

Samples of shrimp and sediment were collected from intensive shrimp ponds using purposive sampling. The sediment samples were collected from the outlet of the pond. From each location, 3 samples from shrimp and sediment were collected. The research location was selected based on its status as a sizable *P. vannamei*-producing area in Central Java. The collected samples were stored in labeled sample bottles and placed in a cool box.

Collected samples were isolated using TCBS agar and Zobell Marine Agar. Water samples were serially diluted in phosphate-buffered saline (PBS). The shrimp and sediment samples were crushed and dissolved in PBS before dilution and spreading. Diluted samples were then cultured using the spread plate method, followed by 1×24 hours of incubation at 37°C. Bacterial colonies that emerged after incubation were selected based on their morphological features.

Antimicrobial susceptibility tests were performed using the Kirby-Bauer method and Clinical and Laboratory Standards Institute (CLSI) guidelines M100 (Bauer *et al*., 1966; CLSI, 2020). The pure isolate obtained was cultured in liquid media (24 h), diluted using a PBS solution until it reached the McFarland standard of 0.5, and then cultured using a cotton swab on Mueller-Hinton Agar media. Antibiotic discs were placed on Mueller-Hinton agar plates that had been cultured (Peña *et al*., 2024; Mumbo *et al*., 2023). The inhibition zones obtained after 24 h of incubation were measured and compared to the CLSI guideline breakpoint. The dose and interpretation level of antibiotic resistance were determined based on the CLSI guidelines (Bauer *et al*., 1966; CLSI, 2020). The interpretation criteria for antimicrobial susceptibility testing followed CLSI guidelines and are presented in Table 1

Bacterial DNA was extracted using the Zymo Bacterial Miniprep Extraction Kit, following the Quick-DNA™ Fungal/Bacterial Miniprep Kit Protocol. The concentration of pure DNA templates was measured using a NanoDrop spectrophotometer before amplification (Sarjito *et al*., 2022). The 16S rRNA genes were amplified using PCR, and a 25 µL master mix was prepared by mixing 2.5 µL of DNA template, 2 µL of each primer, 6 µL of ddH2O, and 12.5 µL GoTaq Green. The primers used were universal primers 27F (5'-AGAGTTTGATC-CTGGCTCAG-3') and 1492R (5'-TAC-GGCTACCTTGT-TACGACTT-3'). The master mix was then run on a SimpliAmp PCR Thermal Cycler following Amalia *et al*. (2024), with the following temperature settings: initial denaturation at 95°C for 1 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 68°C for 1 min; and a final extension at 72°C for 7 min. PCR products were run on a 1X TAE buffer gel at 100 V for 30 min and visualized with Gel Doc E.Box. Amplified bands observed in the gel were sent to 1st BASE for sequencing. Sequence data were compared with the GenBank database at the National Center for Biotechnology Information (NCBI) using BLAST tools. BLAST results were used for the phylogenetic analysis. Sequences were aligned using Clustal W, and a phylogenetic tree was constructed using the neighbor-joining method with the Jones–Taylor–Thornton model in MEGA-X. Bootstrap analysis with 1000 replicates was performed (Saitou *et al*., 1987; Kumar *et al*., 2018).

RESULTS AND DISCUSSION

Twelve shrimp and sediment samples were collected, and the isolation results on TCBS Agar media yielded various bacterial isolates, with the morphological characteristics of each colony presented in Table 2 and Figure 2. From the total isolates obtained, four representative isolates were

selected for further chaacterization based on differences in colony morphology and preliminary antimicrobial resistance screening results. Therefore, the selected isolates were not intended to represent the entire microbial diversity of the ponds, but rather to identify potentially antibiotic-resistant bacteria associated with intensive shrimp aquaculture environments.

Of all the bacterial isolates obtained from the isolation results, four were selected based on their morphological characteristics, specifically those that demonstrated confirmed resistance phenotypes during the preliminary study. All isolates were tested for resistance using the CLSI method, as presented in Table 3. The resistance level, measured by the clear zone diameter on the MHA medium, varied widely.

The data presented in Table 3 show the indication of variations in bacterial resistance levels among the tested isolates. A23 exhibited complete resistance to all antibiotics tested, as indicated by the absence of an inhibition zone (0.00 mm). In contrast, B12 showed a considerable inhibition zone against ERY (27.90 ± 4.38 mm) and moderate against AMX (16.32 ± 0.63 mm), but was absent in OXY and ENR. In contrast, B15 and B17 showed intermediate inhibition across multiple antibiotics, particularly ENR and AMX, suggesting partial susceptibility and class-specific resistance mechanisms. It also means reduced therapeutic response to amoxicillin under standard conditions. In aquaculture, treatment effectiveness may depend on antibiotic concentration, exposure duration, and bacterial load, increasing the risk of failure if antimicrobial use is mismanaged (Thornber *et al*., 2019; Diaz *et al*., 2022). The distinct resistance mechanisms of macrolide (ERY), β-lactam (AMX), and tetracycline also show how they can influence the responses observed across classes in the isolate observed (Hirshfeld *et al*., 2023).

These findings indicate variations in the responses of each antibiotic to isolates commonly found in aquaculture-associated bacterial communities. From Table 3, we can also conclude that variable resistance patterns have been found in aquaculture-associated bacteria across different antibiotics, demonstrating that partial susceptibility profiles are a recognized phenotypic category in aquaculture-associated isolates (Shahadat *et al*., 2025). The resistance level category is presented in Table 4.

Table 2. The morphological characteristics of the bacterial colonies collected from isolated samples

Samples Code	Morphological Characteristics					
	Color	Shape	Margin	Elevation	Opacity	Colony Size
A23	whitish green	circular	entire	convex	translucent	small
B12	whitish green	circular	entire	convex	opaque	small
B15	yellow	irregular	entire	flat	transluscent	large
B17	whitish green	circular	entire	convex	opaque	small

a b c

Figure 2. Morphology of isolates in TCBS agar
Note: a. A23; b. B12; B17; c. B15

The inhibition diameters in Table 4 were classified into sensitivity categories. We found that A23 was categorized as resistant (R) to all antibiotics, confirming that A23 is a multidrug-resistant (MDR) bacterium. Isolate B12 showed susceptibility (S) only to ERY, resistance to OXY and ENR, and intermediate susceptibility (MS) to AMX. In contrast, isolate B15 remained resistant to ERY, OXY, and ENR but was susceptible to AMX. Similar to B15, B17 showed moderate resistance to ENR.

The variability of bacterial resistance was found to be evidence of selective pressure from the use of antibiotics in aquaculture systems, which drives the emergence of antibiotic-resistant bacterial strains (Mohammed *et al*., 2025). As shown in Table 3, oxytetracycline resistance was observed in all isolates. Previous studies have reported that tetracycline resistance is the most prevalent trait in bacteria associated with shrimp (Zhang *et al*., 2024). This statement is supported by data indicating that tetracycline is widely used in global shrimp hatcheries, along with its widespread application in aquaculture (Elbashir *et al*., 2021). High levels of resistance were also observed with β-lactam antibiotics, such as amoxicillin, which is widely found in *Vibrio* spp. (Iftehimul *et al*., 2025). Some still retain susceptibility, as observed in B15. The presence of MDR isolates is particularly concerning, as these bacteria can act as reservoirs of antimicrobial resistance genes (ARGs), facilitating horizontal gene transfer within aquatic environments (Elsabeh *et al*., 2025; Rodríguez-Blanco *et al*., 2025).

The four isolates showed fluorescent bands in the range of 1500bp. The sequencing results were edited in MEGA 11 to remove errors and trim the ends before proceeding with the phylogenetic analysis. The edited DNA was then compared with the NCBI database using BLAST. The results obtained for various bacterial species are presented in Table 5.

The DNA sequences were aligned to create a phylogenetic tree based on their relatedness. The phylogenetic tree is shown in Figure 3. As shown in Table 5 and Figure 3, all isolates had 100% homology with their closest relatives. All these isolates are commonly found in marine environments, including shrimp and pond sediments (Sampaio *et al*., 2022; Zghab, 2025). The most commonly found bacteria in shrimp cultivation activities are of the genus *Vibrio* sp., which are one of the main pathogens for shrimp aquaculture commodities (Ortega *et al*., 2026). Molecular analysis based on 16S rRNA gene sequencing revealed that the isolates belonged to different bacterial taxa. Isolate A23 showed 100% homology to *V. injensis* strain VS1, isolate B15 was identified as *V. metschnikovii*, and B12 and B17 corresponded to *S. arlettae*. The high sequence similarity (100%) indicates accurate taxonomic identification and strong phylogenetic relationships. The predominance of *Vibrio* species (A23 and B15) is consistent with numerous studies reporting *Vibrio spp.* as the dominant bacteria in shrimp aquaculture environments, as bacteria of the genus *Vibrio* are ubiquitous in aquatic environments and include several species pathogenic to both humans and aquatic animals (Hansen *et al*., 2022), with their presence consistently documented across global shrimp farming systems (Vaiyapuri *et al*., 2021; Hirshfeld *et al*., 2023).

These bacteria are well-known opportunistic pathogens responsible for vibriosis and are frequently associated with antimicrobial resistance (AMR). The frequency of antibiotic-resistant *Vibrio* spp. is growing across aquaculture systems, posing a threat to both ecosystem integrity and public health, and related species exhibit high levels of resistance to multiple antibiotics (Ortega *et al*., 2026; Thornber *et al*., 2020). This poses risks to both aquaculture productivity and public health, antibiogram profiles, and the emergence of multidrug-resistant bacteria from farmed shrimp (Devadas *et al*., 2025; Milijasevic *et al*., 2024). The identification of *S. arlettae* (B12) suggests possible contamination from environmental sources, reflecting its broad environmental distribution across terrestrial and aquatic habitats, and it is known to harbor resistance genes (Lavecchia *et al*., 2019). The identification of *S. arlettae* (B12) has not been previously reported from shrimp aquaculture in Indonesia, and constitutes a novel finding. This finding reflects its broad environmental distribution across terrestrial and aquatic habitats and its known capacity to harbor resistance genes, including those conferring resistance to β-lactam antibiotics (Lavecchia *et al*., 2019; Gupta *et al*., 2025). Originally isolated from poultry and goats, strains of this species have since been recovered from diverse environments such as salt mines and estuaries (Lavecchia *et al*., 2019; Pereira and Ramaiah,

2019), and its presence in aquaculture systems may facilitate the dissemination of clinically relevant resistance determinants across bacterial communities (Gupta *et al.*, 2025).

Table 3. Diameter of Inhibition Zone (mm)

Samples Code	Diameter of Inhibition Zone (mm)			
	ERY (15 µg)	OXY (30 µg)	ENR (5 µg)	AMX (20 µg)
A23	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
B12	27.90±4.38	0.00±0.00	0.00±0.00	16.32±0.63
B15	13.42±0.66	9.82±1.05	13.25±1.61	20.92±0.51
B17	12.25±0.35	14.77±0.53	15.57±1.36	20.18±1.16

Table 4. Resistance level category

Samples Code	ERY (15 µg)	OXY (30 µg)	ENR (5 µg)	AMX (20 µg)
A23	R	R	R	R
B12	S	R	R	MS
B15	R	R	R	S
B17	R	R	MS	S

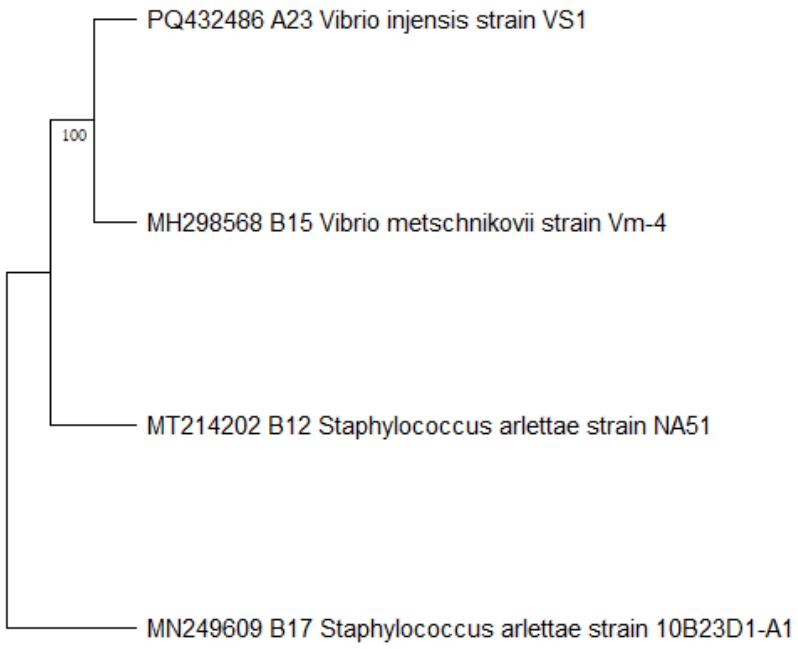

Figure 3. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacterial isolates obtained from intensive shrimp ponds. Bootstrap values were calculated from 1000 replicates.

Table 5. The homology results from isolated samples

Sample code	Length (bp)	Closest Relativity	Homology	Reference accession number
A23	1330	Vibrio injensis strain VS1	100%	PQ432486.1
B12	1403	Staphylococcus arlettae strain NA51	100%	MT214202.1
B15	1389	Vibrio metschnikovii strain Vm-4	100%	MH298568.1
B17	1416	Staphylococcus arlettae strain 10B23D1-A1	100%	MN249609.1

Overall, bacterial isolates associated with intensive shrimp aquaculture exhibit resistance to multiple antibiotic classes. *Vibrio* was the most frequently identified genus, consistent with its established role as a dominant opportunistic pathogen in marine and brackish water aquaculture systems. This intraspecies variability reflects the heterogeneous selective pressure operating within the intensive pond environment and is consistent with the documented capacity of environmental staphylococcal populations to independently acquire and express resistance determinants across individual strains.

CONCLUSIONS

This study confirmed the presence of diverse multidrug-resistant bacterial assemblages, comprising *V. injensis, V. metschnikovii*, and *S. arlettae*, in intensive *P. vannamei* pond environments in Kendal Regency, Central Java, Indonesia. Universal oxytetracycline resistance across all isolates and complete MDR in V. injensis collectively indicate that intensive shrimp pond systems in this region function as active reservoirs for the emergence and dissemination of antimicrobial resistance. The identification of *S. arlettae* — not previously documented in Indonesian intensive shrimp aquaculture — further underscores the role of opportunistic environmental bacteria as potential vectors for resistance gene dissemination within pond-associated microbial communities. These findings highlight the urgent need for systematic AMR surveillance, responsible antibiotic stewardship, and alternative disease management strategies to mitigate the escalating risk of multidrug resistance in Indonesian shrimp aquaculture.

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