In-Vitro Studies on Antimicrobial Susceptibility and Multiple Antibiotic Resistance Profile of Vibrio parahaemolyticus Isolated from Market Shrimp in Andhra Pradesh, India

Kopuri. Aruna*[1], T. Sujatha[1], N. Lakshmi Janaki[2], Talluri. Madhu Mohan[3] and Vemuri. Praveen Kumar[4]

[1]Department of Microbiology, S.R.R & C.V.R Govt. Degree College (A) College, Vijayawada-520 004, Andhra Pradesh, India.

[2]Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, and S.R.R & C.V.R Govt. Degree College (A), Vijayawada-520 004, Andhra Pradesh, India.

[3]Department of Biotechnology, Koneru Lakshmaiah University, (Deemed to be University estd., u/s 3 of UGC Act 1956) Vaddeswaram, Guntur (Dist.) Andhra Pradesh - 522502, India and SGS Aqua Solutions, 68-6-18B, R-SQUARE residence Ashok Nagar, Kakinada-533 004, Andhra Pradesh, India.

[4]Department of Biotechnology, Koneru Lakshmaiah University, (Deemed to be University estd., u/s 3 of UGC Act 1956) Vaddeswaram, Guntur (Dist.) Andhra Pradesh - 522502, India.

***Corresponding Author**

DOI: https://doi.org/10.51244/IJRSI.2026.1307000355

Received: 06 August 2026; Accepted: 11 August 2026; Published: 19 August 2026

ABSTRACT

Vibrio parahaemolyticus is a halophilic, Gram-negative marine pathogen and a leading cause of seafood-associated gastroenteritis worldwide. Rising seafood consumption, together with unregulated antibiotic use in aquaculture, has driven the emergence of multidrug-resistant strains, raising both clinical and food-safety concerns. In the present study, V. parahaemolyticus was isolated from the hepatopancreas of market shrimp collected from Andhra Pradesh, India, and confirmed by a standard biochemical panel. Antimicrobial susceptibility was evaluated against nine commercially available antibiotics such as streptomycin, ampicillin, gentamycin, tetracycline, cephalexin, ciprofloxacin, neomycin, kanamycin and ceftriaxone using the agar well-diffusion method, and the minimum inhibitory concentration (MIC) of each antibiotic was determined by broth microdilution in 96-well microtiter plates. The isolate produced the largest overall zone of inhibition with ciprofloxacin (52.6 mm at 100 µg) and the smallest zone among all tested antibiotics with cephalexin (18.6 mm at 25 µg), while gentamycin produced the largest zone (29 mm) at the 25 µg concentration. Correspondingly, ciprofloxacin (7.812 µg/mL) and gentamycin (9.114 µg/mL) recorded the lowest MIC values, whereas ampicillin (333.33 µg/mL), cephalexin (250 µg/mL) and ceftriaxone (166 µg/mL) required considerably higher concentrations for inhibition, indicating comparative resistance. The multiple antibiotic resistance (MAR) index of the isolate was 0.75, well above the 0.2 threshold that flags isolates from environments with heavy antibiotic exposure. These findings confirm that V. parahaemolyticus from retail shrimp harbours resistance to several clinically and agriculturally important antibiotics and underscore the need for continued antimicrobial surveillance of seafood in this region.

Keywords: Vibrio parahaemolyticus; antimicrobial susceptibility; well diffusion assay; minimum inhibitory concentration; multiple antibiotic resistance index; shrimp.

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative, halophilic member of the family Vibrionaceae that is widely distributed across estuarine, marine, and coastal ecosystems. It typically exists in a free-living, motile state, propelled by a single polar flagellum, and readily attaches to inert and living surfaces such as zooplankton, finfish, shellfish, and other suspended organic matter. Strains are classified according to the antigenic

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue VII July 2026

properties of their somatic (O) and capsular (K) antigens, which vary with environmental conditions. The organism was first identified as a human pathogen by Tsunesaburo Fujino in 1950, following a large food-poisoning outbreak in Japan linked to the consumption of shirasu (semi-dried sardines) that resulted in 272 illnesses and 20 deaths (Jiang et al., 2014; Silvester et al., 2021).

Parahaemolyticus naturally proliferates in warm coastal waters, and its abundance peaks during the summer months. Global warming has progressively raised sea-surface temperatures, extending the geographic and seasonal window in which the organism can establish itself, including in waters where it was previously undetected (Lee et al., 2018). Human infection follows the consumption of raw or undercooked fish, crustaceans, and molluscs, and typically manifests as self-limiting gastroenteritis with watery diarrhoea, nausea, and abdominal cramps; in immunocompromised individuals, however, infection can progress to septicaemia and death. V. parahaemolyticus remains one of the leading causes of seafood-borne illness in Asia and the United States, and its incidence in Europe is rising in step with increasing seafood consumption and warming coastal waters (Yang et al., 2017).

Pathogenicity in V. parahaemolyticus is closely linked to the production of two pore-forming haemolysins, thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH), encoded by the tdh and trh genes, respectively; strains carrying trh frequently also produce urease, which aids intestinal colonisation and cytokine activation. Because most infections are self-limiting, antimicrobial therapy is reserved for severe or invasive cases, where cephalosporins, tetracyclines, quinolones, and fluoroquinolones are the agents of choice (Li et al., 2017; Letchumanan et al., 2015). However, the same antibiotic classes — together with penicillins and aminoglycosides — are used extensively, and often without adequate regulation, in aquaculture and shellfish farming to control bacterial disease and to promote growth. This dual clinical and agricultural exposure has driven the steady emergence of antimicrobial-resistant and multidrug-resistant V. parahaemolyticus strains across Asia, the Americas, and Europe (Letchumanan et al., 2014).

In India, V. parahaemolyticus has been recovered from both clinical and environmental sources, including diarrhoeal patients, urban-slum outbreaks, and retail seafood from fishing landings in southern and eastern coastal states, with reports of multidrug-resistant and ampicillin-resistant isolates from several markets (Jiang et al., 2014; Yang et al., 2017). Because retail shrimp is harvested from waters and farming systems subject to variable, and often uncontrolled, antibiotic pressure, and because it enters the human food chain with minimal further processing, it represents both a sensitive indicator of environmental antimicrobial pressure and a plausible route for the transfer of resistant strains and resistance genes to humans.

Despite this, systematic, up-to-date antibiograms for V. parahaemolyticus isolated from retail shrimp sold in local Indian markets remain limited, particularly studies that combine qualitative (zone-of-inhibition) and quantitative (MIC) susceptibility data for a broad panel of clinically and agriculturally relevant antibiotics on the same isolate. Such combined data are important for two reasons. First, they allow the treating clinician or aquaculture veterinarian to identify which first-line antibiotics remain effective. Second, they generate the baseline surveillance data needed to track the trajectory of resistance over time and to inform the regulation of antibiotic use in shrimp farming and marketing. Generating this evidence for a locally sourced isolate therefore has direct relevance to both public health and food-safety policy in the region, and is the central motivation for the present work.

Therefore, the present study was designed with the following objectives: (i) to isolate V. parahaemolyticus from market shrimp collected in Andhra Pradesh, India; (ii) to determine the antimicrobial activity of nine commercially available antibiotics against the isolate using the agar well-diffusion assay; and (iii) to determine the minimum inhibitory concentration of each antibiotic against the isolate using the broth microdilution method.

MATERIALS AND METHODS

All reagents used were of analytical grade and were used as supplied, without further purification. Streptomycin, ampicillin, gentamycin, tetracycline, cephalexin, ciprofloxacin, neomycin, kanamycin, and ceftriaxone were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

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Isolation of V. parahaemolyticus:

Fresh shrimp were purchased from a local market in Andhra Pradesh, India, and rinsed with 0.9% saline. The hepatopancreas was aseptically dissected, homogenised in phosphate-buffered saline (pH 7.4), and serially diluted in 0.9% saline. Aliquots were spread on thiosulfate–citrate–bile salts–sucrose (TCBS) agar and incubated at 37 °C for 24 h. Presumptive colonies were repeatedly sub-cultured to purity and confirmed as V. parahaemolyticus using a standard biochemical identification panel (HiMedia).

Agar well-diffusion assay:

Mueller–Hinton agar (beef infusion 300 g, casein hydrolysate 17.5 g, starch 15 g, and agar 17 g per litre of distilled water; pH 7.0–7.2) was autoclaved (121 °C, 15 psi, 15 min) and poured into sterile Petri dishes. Plates were swabbed uniformly with a standardised inoculum (~10^5 CFU/mL) of the isolate using a sterile cotton swab, and six wells (2.5 mm depth) were punched into each plate with a sterile gel puncture. The central control well received 50 µL of 10% phosphate-buffered saline, while the remaining wells were loaded with 25, 50, 75, and 100 µg of each of the nine test antibiotics. Plates were incubated at 25 °C for 24 h under aseptic conditions, and zones of inhibition were measured in triplicate; results are expressed as mean ± SD.

Minimum inhibitory concentration:

MIC was determined in sterile 96-well microtiter plates by two-fold serial dilution of each antibiotic in Mueller–Hinton broth inoculated with a standardised bacterial suspension (~10^5 CFU/mL). Columns 11 and 12 served as sterility and growth controls, respectively. Plates were incubated at 25 °C for 24 h and examined visually; turbidity indicated growth and clarity indicated inhibition. The lowest concentration producing no visible turbidity was recorded as the MIC. Assays were performed in triplicate, and results are expressed as mean ± SD. The multiple antibiotic resistance (MAR) index of the isolate was calculated as MAR = a/b, where a is the number of antibiotics to which the isolate showed resistance and b is the total number of antibiotics tested (Krumperman, 1983).

RESULTS AND DISCUSSION

Confirmation of the isolate

Colonies recovered from TCBS agar were green, round, and 2–3 mm in diameter, consistent with V. parahaemolyticus. Biochemical testing (Table 1) confirmed the identification: the isolate was positive for arginine utilisation and salt tolerance, and negative for the Voges–Proskauer reaction, ONPG, citrate utilisation, ornithine utilisation, and fermentation of mannitol, arabinose, sucrose, glucose, salicin, and cellobiose. This biochemical profile is typical of V. parahaemolyticus and matched the expected reference pattern for the species.

Table 1. Biochemical confirmation of the Vibrio parahaemolyticus isolate.

No.	Biochemical test	Colour observed	Interpretation
1	Voges–Proskauer (VP)	Brown / copper	**Negative (−)**
2	Arginine utilisation	Dark brown / purple	**Positive (+)**
3	Salt tolerance	Dark green	**Positive (+)**

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4	ONPG	Cream / colourless	**Negative (−)**
5	Citrate utilisation	Dark green	**Negative (−)**
6	Ornithine utilisation	Brown	**Negative (−)**
7	Mannitol	Red / orange	**Negative (−)**
8	Arabinose	Red / orange	**Negative (−)**
9	Sucrose	Red / orange	**Negative (−)**
10	Glucose	Red / orange	**Negative (−)**
11	Salicin	Red / orange	**Negative (−)**
12	Cellobiose	Pink / red	**Negative (−)**

Agar well-diffusion assay

Figure 1 shows representative Petri dish plates for each of the nine antibiotics tested against the isolate at 25, 50, 75, and 100 μg per well, alongside the phosphate-buffered saline control. Clear, dose-dependent zones of inhibition were visible around every antibiotic well, while the control wells showed no inhibition, confirming that the observed zones were antibiotic-specific and that the isolate was susceptible, to varying degrees, to all nine antibiotics tested.

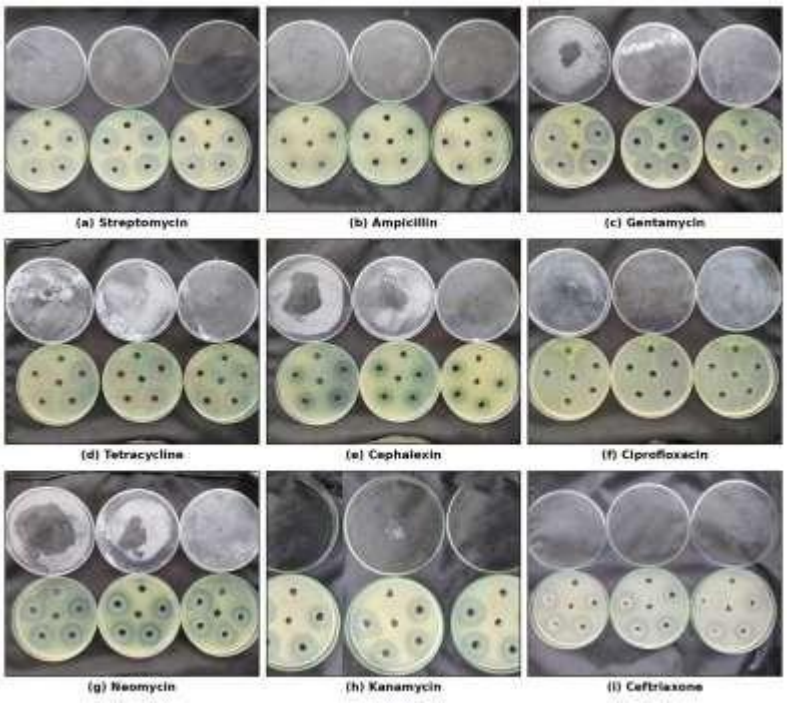

Figure 1. Agar well-diffusion assay of Vibrio parahaemolyticus against nine antibiotics. Top row of each panel: uninoculated / control comparison plates; bottom row: inoculated Mueller–Hinton agar plates

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showing wells loaded with the control (PBS) and increasing antibiotic concentrations (25–100 µg), with zones of inhibition visible around each drug-loaded well.

Among the nine antibiotics, cephalexin produced the smallest zone of inhibition recorded in the study (18.6 mm at 25 µg), indicating comparatively weak activity against the isolate at this concentration. Gentamycin produced the largest zone at the same 25 µg concentration (29 mm), and ciprofloxacin produced the largest zone overall across all concentrations tested (52.6 mm at 100 µg) (Figure 2). These results indicate that ciprofloxacin and gentamycin were the most effective antibiotics against the isolate in the diffusion assay, while cephalexin was comparatively the least effective at the lowest concentration tested.

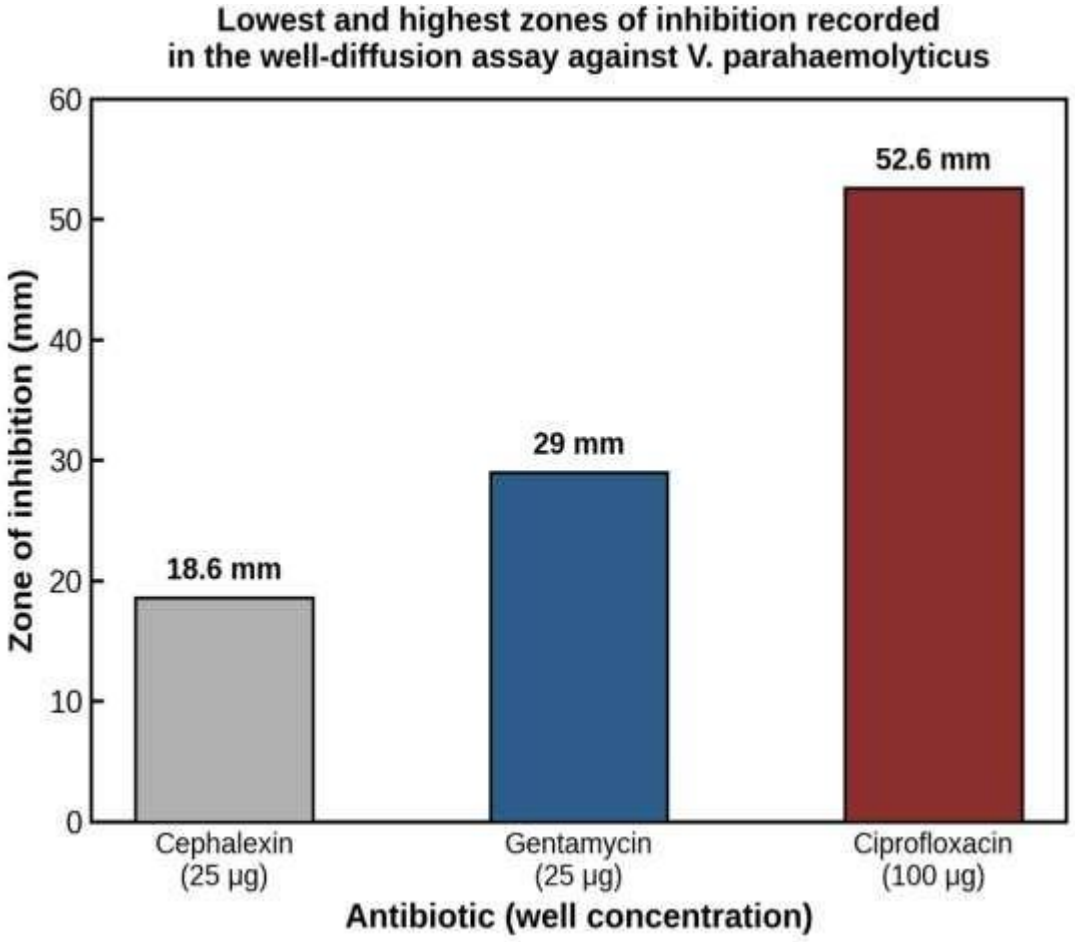

Figure 2. Lowest and highest zones of inhibition recorded against Vibrio parahaemolyticus in the well-diffusion assay, shown with the corresponding well concentration.

Minimum inhibitory concentration

The MIC of each antibiotic was determined by broth microdilution in 96-well microtiter plates (Figure 3). Wells with visible turbidity were scored as bacterial growth, and clear wells were scored as inhibition. Ciprofloxacin exhibited the lowest MIC (7.812 µg/mL), followed by gentamycin (9.114 µg/mL), confirming these as the two most potent antibiotics against the isolate. In contrast, considerably higher concentrations were required to inhibit growth with streptomycin and tetracycline (125 µg/mL each), ceftriaxone (166 µg/mL), cephalexin (250 µg/mL), and ampicillin (333.33 µg/mL) (Figure 4). The comparatively high MIC values for these five antibiotics indicate reduced susceptibility of the isolate, consistent with the pattern observed in the well-diffusion assay. MIC values for neomycin and kanamycin could not be resolved to a single concentration from the available dataset and are reported as not determined (ND).

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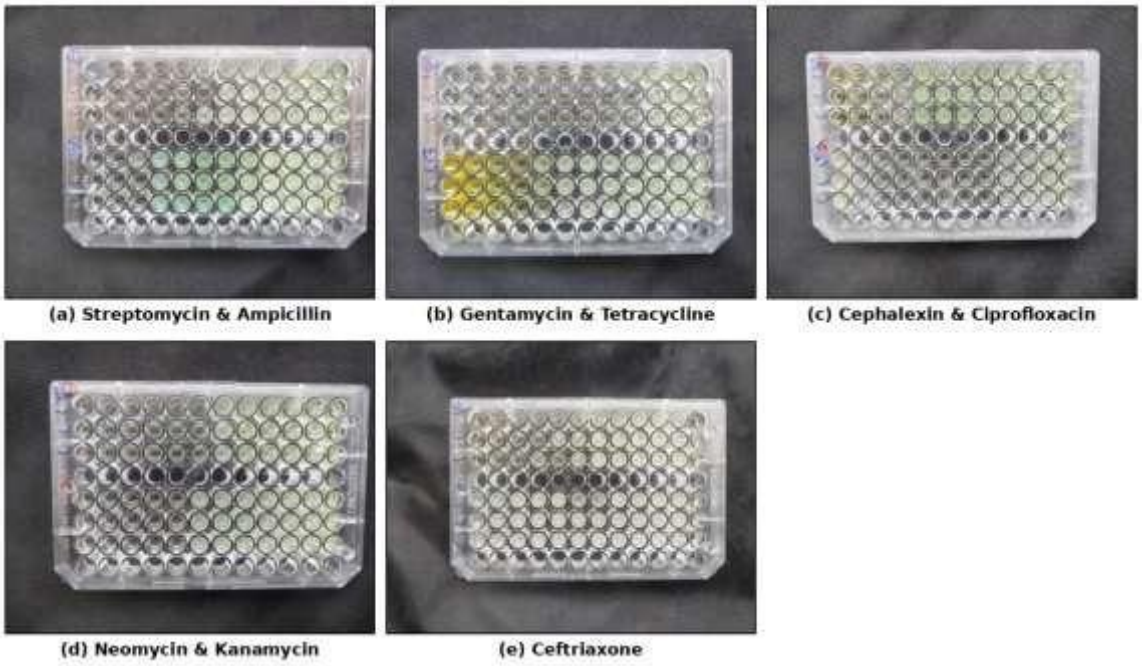

Figure 3. Broth microdilution MIC assay of Vibrio parahaemolyticus against nine antibiotics in 96-well microtiter plates. Turbid wells indicate bacterial growth; clear wells indicate inhibition. Columns 11 and 12 of each plate served as sterility and growth controls, respectively.

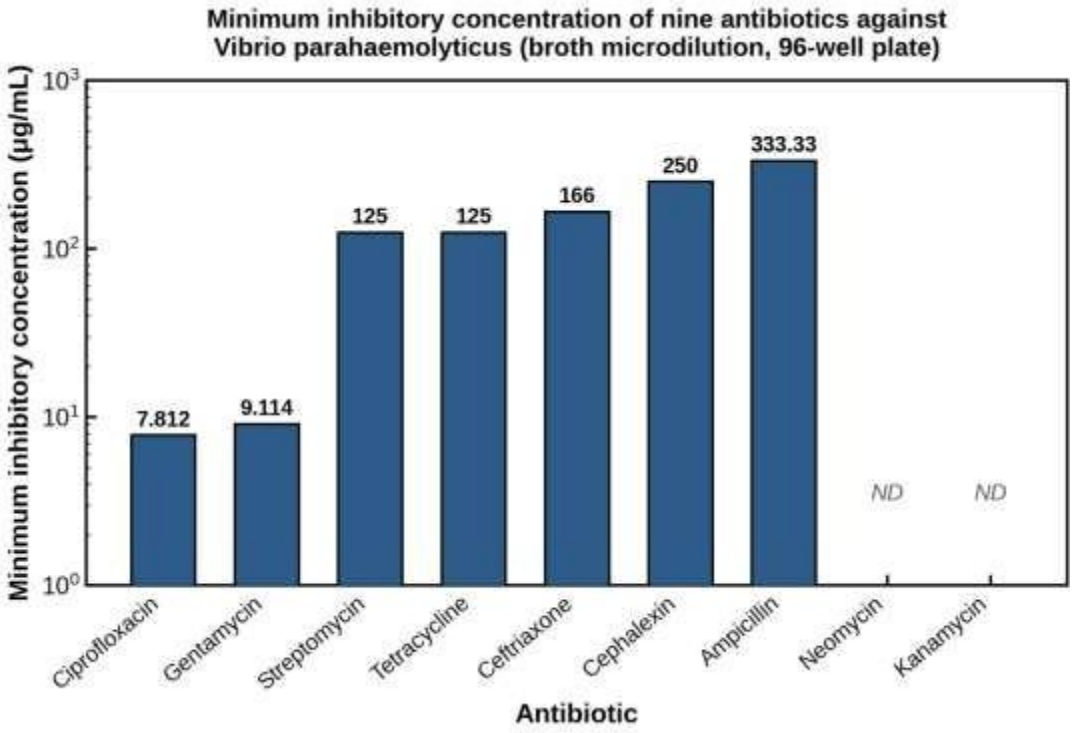

Figure 4. Minimum inhibitory concentration (log scale) of nine antibiotics against Vibrio parahaemolyticus. ND = not determined.

Multiple antibiotic resistance (MAR) index

Based on the panel of nine antibiotics tested, the isolate showed reduced susceptibility toward six antibiotics, giving a MAR index of 0.75. This value is well above the 0.2 threshold historically used to distinguish isolates originating from high-antibiotic-use environments from those with low antibiotic exposure (Krumperman, 1983).

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DISCUSSION

Continuous, and often unregulated, antibiotic use in the aquaculture and shellfish-farming industry is widely regarded as the principal driver of antimicrobial resistance in environmental and food-associated Vibrio populations, since it creates sustained selective pressure that favours resistant clones and promotes horizontal transfer of resistance genes within bacterial communities (Elmahdi et al., 2016; Rebouças et al., 2011). All nine antibiotics evaluated in the present study such as tetracycline, ampicillin, gentamycin, ciprofloxacin, cephalexin, neomycin, kanamycin, streptomycin, and ceftriaxone — are among the agents recommended for treating Vibrio infections in humans and in cultured shellfish and finfish (Shaw et al., 2014), and several, including tetracycline and ampicillin, are also used routinely as prophylactic and growth-promoting agents in shrimp and fish farming (Letchumanan et al., 2014).

The comparatively high MIC of ampicillin (333.33 μg/mL) observed in the present isolate is consistent with numerous reports of ampicillin resistance in V. parahaemolyticus recovered from seafood, including isolates from Malaysian shellfish (Letchumanan et al., 2015) and from Korean and Chinese retail seafood, where ampicillin resistance frequently exceeds 80–90% of tested isolates (Jun et al., 2012; Yang et al., 2017; Lee et al., 2025). Because first-generation β-lactams such as ampicillin have long been used extensively in aquaculture, sustained selective pressure has progressively lowered susceptibility and reduced their clinical utility against Vibrio infections (Sudha et al., 2014).

Reduced susceptibility to the cephalosporins cephalexin and ceftriaxone was also observed in the present isolate. This finding is broadly consistent with Jun et al. (2012), who reported resistance to third-generation cephalosporins among 70–80% of V. parahaemolyticus isolates from Korean seafood, and with reports of cephalosporin- and quinolone-resistant isolates from Malaysian shellfish (Sahilah et al., 2014, as cited in Letchumanan et al., 2015). By contrast, Shaw et al. (2014) reported a comparatively low prevalence of cephalosporin resistance among V. parahaemolyticus isolated from Chesapeake Bay, USA. These inconsistencies across studies are most plausibly explained by differences in testing methodology, breakpoint interpretation, and geographic variation in antibiotic-use practice, and they reinforce the importance of locally generated antibiograms rather than extrapolation from studies conducted elsewhere.

The two most potent antibiotics identified in the present study, ciprofloxacin (MIC 7.812 μg/mL) and gentamycin (MIC 9.114 μg/mL), remain broadly effective against V. parahaemolyticus in several recent surveys (Letchumanan et al., 2015; Yang et al., 2017). However, sub-lethal exposure to fluoroquinolones and aminoglycosides has been shown to select for resistant sub-populations over time through point mutations and adaptive metabolic changes, indicating that even currently effective antibiotics are not immune to future resistance development if usage in the food chain remains uncontrolled (Yang et al., 2024).

The MAR index of 0.75 recorded for the present isolate is markedly higher than the 0.2 threshold proposed by Krumperman (1983) to flag isolates from high-antibiotic-use environments, and is also higher than MAR indices reported for V. parahaemolyticus from several other regional seafood surveys, which have generally ranged between 0.21 and 0.79 depending on the sampling source and the antibiotic panel tested (Lee et al., 2018; Silvester et al., 2021). This comparatively high value suggests that the shrimp sampled in the present study originated from a supply chain subject to substantial antibiotic exposure, and it is consistent with the broader pattern of rising multidrug resistance reported among V. parahaemolyticus isolated from Indian and other Asian seafood markets over the past decade (Jiang et al., 2014; Li et al., 2017). Continued surveillance of retail seafood, together with more prudent regulation of antibiotic use in aquaculture, will be necessary to preserve the clinical utility of first-line antibiotics against this pathogen.

CONCLUSION

The V. parahaemolyticus isolate recovered from market shrimp in Andhra Pradesh, India, was confirmed biochemically and displayed a mixed antimicrobial susceptibility profile against nine commercially available antibiotics. Ciprofloxacin and gentamycin were the most effective agents, with the lowest MIC

values and the largest zones of inhibition, whereas ampicillin, cephalexin, ceftriaxone, tetracycline, and streptomycin required substantially higher concentrations for inhibition. The multiple antibiotic resistance index of 0.75 places this isolate well above the recognised high-risk threshold, indicating that it most likely originated from an environment subject to considerable antibiotic pressure. These findings highlight the risk that antibiotic-resistant V. parahaemolyticus in retail shrimp poses to consumers and to the future clinical management of Vibrio infections, and they support the need for regular antimicrobial surveillance of seafood together with more judicious use of antibiotics in shrimp aquaculture and post-harvest handling.

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