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Research Paper

Exploration of Shrimp and Their Environments for the Detection of Antibiotic Resistance Genes of *Vibrio parahaemolyticus* and Spectrophotometry of Shrimp Muscles for Heavy Metals and Their Human Health Risk Assessment in Bangladesh

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A R T I C L E I N F O

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A B S T R A C T

Through deteriorating the quality of shrimp, *Vibrio parahaemolyticus* and heavy metals have become threatened to food safety. The study was conducted to explore shrimp and their environments for antibiotic resistance genes of *V. parahaemolyticus* and perform spectrophotometry of shrimp muscles for heavy metals and their human health risk assessment. In total, 130 samples (shrimp, water, and sediment) were aseptically collected from 27 ponds in four areas of Khulna and Satkhira districts where the number of water and sediments was corresponded to the number of ponds and the number of shrimps differed from pond to pond. *V. parahaemolyticus* were detected by cultural, staining, biochemical, and molecular techniques targeting *groEL*, *tetA*, *tetB*, *tetC*, and bla_{TEM} genes. Disc diffusion assay and bivariate analysis were performed for investigating antibiotic resistance profiles of *V. parahaemolyticus*. Cadmium, chromium, lead, zinc, and iron were measured by AAS (atomic absorption spectrometry) in shrimp. Among 39 isolates (23 from shrimp, 7 from water, 9 from sediment), real-time PCR (polymerase chain reaction) detected 20 of 27 as positive for *groEL*, 12 of 20 for *tetA*, 13 for *tetB*, 12 for *tetC*, and 1 for bla_{TEM}. *V. parahaemolyticus* were highly resistant to tetracycline and ampicillin. Bivariate analysis revealed a significant correlation between the antibiotics. A total of 51.28% of isolates were MDR (multidrug resistant), and the MAR (multiple antibiotic resistance) indices ranged from 0.08 to 0.6. The highest average concentration for Cd was in Debhata, Pb in Dumuria, Cr in Kaliganj, Zn and Fe in Satkhira Sadar. THQ (target hazard quotients) of >1 for Fe in all sampling sites showed a higher level of HI (hazard index). No determined TR (target cancer risk) value exceeded the recommended value ($<10^{-4}$). The study emphasizes the significance of adopting extensive surveillance and monitoring of a large number of shrimp farms for effective antibiotic management and sustainable shrimp production.

In Bangladesh, the aquaculture sector has grown as a leading food production sector, assisting to national economy, animal protein consumption, employment opportunity for rural and farming families, foreign income, maintaining the diversity of the aquatic sector and uplifting the development of socio-economic conditions. According to the Department of Fisheries (DoF), it contributes 2.43% to national GDP (gross domestic product), 22.14% to agricultural GDP, and 1.05% to foreign exchange earnings by exporting fish and fish products in

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2021–2022 (DoF, 2024). Shrimps are one of the major aquaculture commodities sharing the major portion of the global economy and fishery trade. Gher farming of shrimps has been extending in coastal areas. Due to improper and unhygienic conditions, contamination of shrimps can arise at the time of their cultivation, processing, preservation, and storage (Bedane et al., 2022). As a consequence, different *Vibrio* species can infect shrimp that contribute to food spoilage and spread of foodborne diseases (Dutta et al., 2021). In aquatic systems, various anthropogenic practices, including agricultural activities, landfill erosions, embarkation and docking activities, industrial and domestic wastewater as well as natural processes are the sources of heavy metals accumulation (Ezemonye et al., 2019). Shrimp is a top trencherman in aquatic food chain, so it is usually more susceptible to the aggregation of heavy metals from different origins including water, sediments, and foods (Ezemonye et al., 2019).

V. parahaemolyticus is a Gram-negative, halophilic, facultatively anaerobic, nonspore forming, curved, or straight rod-shaped bacterium (Chimalapati et al., 2020). *V. parahaemolyticus* is generally available in tropical and temperate estuarine, marine and costal environments, as well as in shrimp aquaculture worldwide (Manchanayake et al., 2023)

Antimicrobial agents are commonly employed in the shrimp feed, and water not only to improve growth but also to impede and treat bacterial infections (Okeke et al., 2022). Indiscriminate application of antimicrobial agents leads to the advent of antibioticresistant bacteria, and genes like β-lactam and penicillin-resistant gene bla_{TEM-1}, tetracycline-resistant genes *tetA, tetB, tetC*, and many more (Sharma et al., 2021) in shrimp where they are transmitted to human, and human microbes either through food consumption or by mobile genetic elements and may become risky to human health. (Dutta et al., 2021).

Heavy metals can cause liver disorders, cardiovascular anomalies, kidney failure, and death in case of extreme situations besides carcinogenic effects when it crosses the maximum tolerable limits along with several negative effects on the natural balances of the ecosystem (Mokarram et al., 2020). Although FAO and WHO (FAO/WHO, 1984) defined the maximum recommended limits for each heavy metal (Table 1), these recommended values merely can not measure the probabilistic carcinogenic and noncarcinogenic human health risks. Thus, the US Environmental Protection Agency established quantitative frameworks for quantifying potential HI and TR caused by heavy metals (USEPA, 2009).

Various conventional methods like cultivation in TCBS (Thiosulfate-Citrate-Bile Salts-Sucrose) agar are used to detect and quantify *V. parahaemolyticus* in shrimp samples (Costa et al., 2022). To avoid possible bias, the molecular approach with real-time PCR (qPCR) can be applied to identify the target sequence that can be genus or even species-specific and quantify *V. parahaemolyticus* in shrimp (Costa et al., 2022). AAS method can be used to determine the level of heavy metals (mg/kg) in shrimps. Besides comparing with the maximum limits recommended by FAO and WHO (FAO/WHO, 1984), probabilistic THQ, HI, and TR can also be enumerated to interpret whether shrimps from the study areas are safe for human consumption or not. However, to the best of our knowledge, by reviewing other literatures and searching through internet, we could not find out any document relevant to the detection of antibiotic resistance genes of *V. parahaemolyticus* from shrimp and their environments along with spectrophotometry of shrimp muscles for heavy metals and their human health risk assessment in the world. Therefore, the present research work was conducted to explore shrimp and their environments for the detection of antibiotic resistance genes of *V. parahaemolyticus* and to perform spectrophotometry of shrimp muscles for determining the concentration of heavy metals and their human health risk assessment in Bangladesh.

Table 1
Maximum recommended limits of heavy metals for human consumption defined by WHO and FAO for fish, Crustaceans, and Molluscs (FAO/WHO, 1984)

Heavy metals	Maximum recommended limits for human consumption (mg/kg)
Cadmium	1
Chromium	0.05
Lead	2
Iron	100
Zinc	100

Materials and methods

Sampling areas. The south-western region of Bangladesh like Khulna and Satkhira districts is recognized as the leading shrimp farming areas with the yearly average temperature ranging from 24.48 °C to 34.25 °C in Khulna and from 12.5 °C to 35.5 °C in Satkhira. Twenty-seven shrimp culture ponds in four selected areas of Khulna and Satkhira districts were selected for sample collection where 11 ponds were from Khulna district (all ponds from Dumuria upazila) and 16 ponds from Satkhira district (4 ponds from Satkhira Sadar upazila, 6 ponds from each Debhata and Kaliganj upazila) (Fig. 1).

Sampling. Maintaining seven days interval of each sampling, four samplings were done from 27 ponds between November 15 and December 15 in 2023. In Bangladesh, from June to October, the average temperature is 25–35 °C which is almost similar to the optimum temperature for shrimp culture (28–32 °C). As a result, most of the shrimp farmers culture shrimp at this time and the peak harvesting time of shrimp in Bangladesh is November to December. That's why we collected the samples at that time. A total of 130 samples (76 shrimp samples both healthy and unhealthy, 27 water samples, and 27 sediment samples) were randomly collected within that time period. Among 130 samples, we collected 17 from Satkhira Sadar (9 shrimp samples, 4 water samples, and 4 sediment samples), 41 from Dumuria (19 shrimp samples, 11 water samples, and 11 sediment samples), 52 from Kaliganj (40 shrimp samples, 6 water samples, and 6 sediment samples), and 20 from Debhata (8 shrimp samples, 6 water samples, and 6 sediment samples). As the target organism *V. parahaemolyticus* cannot grow in freshwater, so the salinity of pond water was checked and it was recorded as 7 ppt on average (Jones & Summer-Brason, 1998; DePaola et al., 2003). To collect shrimp samples, cast-net and dragnet were used. Separate sterile plastic zipper bags were taken for each shrimp sample to skip cross-contamination. Sterile Falcon tubes and polythene bags were used for water and sediment samples respectively. Aseptic condition was maintained during sample collection. Labelling was done on all the zipper bags, Falcon tubes, and polythene bags indicating code number, type of the sample, date of collection, and location of sampling. Then, all the samples were immediately placed in the ice box and carried to the laboratory of the Department of Microbiology and Public Health at Khulna Agricultural University for further processing and analysis. If 1 of 3 collected samples from a certain pond tested positive for *V. parahaemolyticus*, that pond was marked as contaminated with bacteria.

Sample processing. Dilution of water and sediment samples was done by tenfold dilution using 0.1% peptone water. Then, 100 μL of these two types of samples were inoculated into alkaline peptone water with a pH level between pH 8.5-pH 9 and a high concentration of NaCl since the target organism *V. parahaemolyticus* is halophilic in nature (Farmer et al., 2003; DePaola & Kaysner, 2004). Sterile distilled water was used to thoroughly wash the shrimp samples, and then, dissection was done by using sterile scissors. Three types of samples namely gill, hepatopancreas, and muscle were collected aseptically,

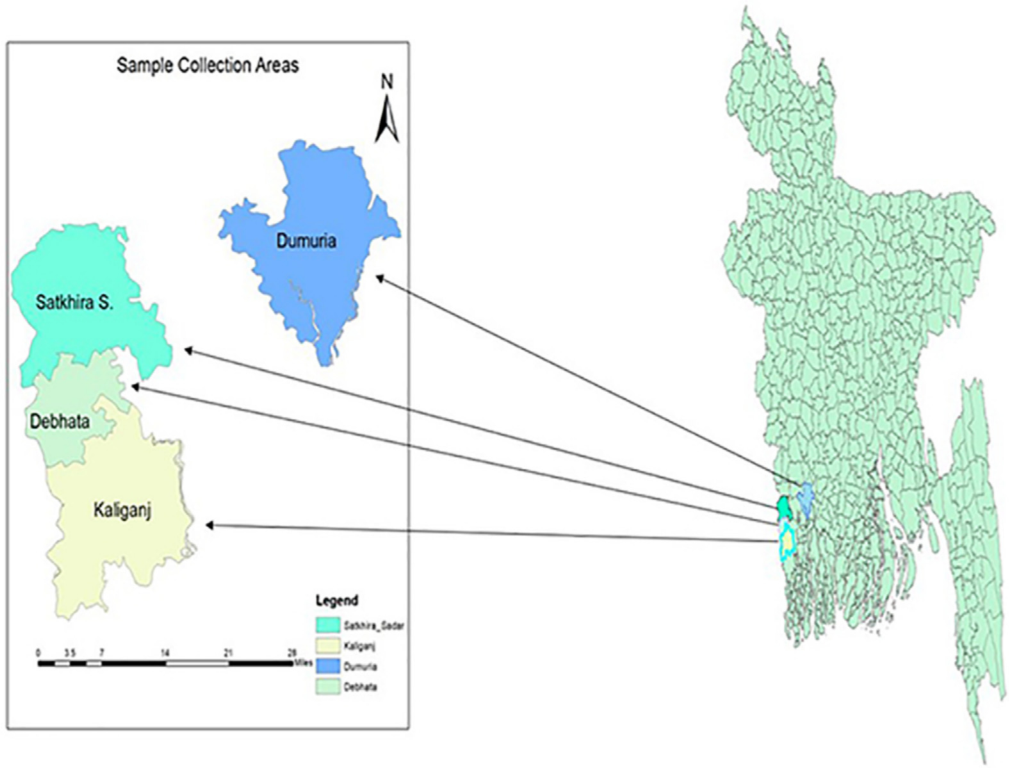

Figure 1. Sampling areas, developed by ArcMap 10.5 (ArcGIS Enterprise, ESRI, Redlands, CA, USA).

and then, the samples were blended and taken into alkaline peptone water for enrichment (Farmer et al., 2003; DePaola & Kaysner, 2004). After that, all the samples including shrimp, water, and sediment were kept in the incubator overnight at 37 ˚C for bacterial enrichment. For heavy metals analysis, approximately 100 g of edible muscle was taken in a clean brown envelop from 20 shrimp samples. Then, they were placed in a hot-air oven to dry at 105 ˚C for 24 h. After that, a grinder was used to crumble the samples. Before using all the glass equipment, they were kept in diluted HNO_3 for 24 h, and then, distilled water was used to wash them.

Cultural characterization of *V. parahaemolyticus*. A total of 100 μL of enriched culture was spread on a previously prepared Plate count agar (PCA) plate (Liofilchem, Italy) by using a sterile glass rod. After overnight incubation at 37 ^{0}C, colony counting was done. The plate containing 30–300 colonies was selected for the calculation of colony forming unit (CFU). After that, a single colony was picked with an inoculating loop and streaking was done on a previously prepared TCBS agar plate (HiMedia, India), and incubation was done overnight at 37 ˚C as already reported (FAO/WHO, 2016). Then, bacterial colonies that were tentatively identified as *V. parahaemolyticus* were streaked again onto newly prepared TCBS agar plates by sterile inoculating loop for further purification.

Gram's staining of *V. parahaemolyticus*. Colonies suspected for *V. parahaemolyticus* were Gram-stained to ascertain their morphology according to the method described by Cheesbrough (2005). All the reagents were brought from an Italian company, Liofilchem.

Biochemical characterization of *V. parahaemolyticus*. According to the protocol stated in Bergey's Manual of Determinative Bacteriology (Bergey et al., 1974), biochemical assays like indole test, catalase test, oxidase test, and nitrate reduction test were conducted. All the reagents were brought from German company, Merck.

Molecular detection of *V. parahaemolyticus*. Final affirmation of *V. parahaemolyticus* was performed by real-time PCR assay focusing on the species-specific *groEL* gene since it has been suggested as a suitable marker for the detection of many bacterial pathogens including *Vibrio* species efficiently and reliably (Hossain et al., 2012a, 2012b, 2013a, 2013b; Ren & Hill, 2023) (Table 2). Since most frequently observed antibiotic resistance profiles of *V. parahaemolyticus* involved ampicillin, penicillin, and tetracycline regardless of the countries, antibiotic resistance *tetA, tetB, tetC,* and bla_{TEM} genes were targeted to assess their genotype (Elmahdi et al., 2016) (Table 2).

Table 2
Forward and reverse primers used for characterization of *V. parahaemolyticus*

Target genes	Primer sequence (5′→3′)	Reference
groEL	F-GTCAGGCTAAGCGCGTAAGCA R-GCATGCCTGCGCTTTCTTTTTG	Hossain et al. (2013)
tetA	F-GTAATTCTGAGCACTGTCGC R-CTGCCTGGACAACATTGCTT	Kim et al. (2013)
tetB	F-ACGTTACTCGATGCCAT R-AGCACTTGTCTCCTGTT	Kim et al. (2013)
tetC	F-AACAATGCGCTCATCGT R-GGAGGCAGACAAGGTAT	Kim et al. (2013)
bla_{TEM}	F-ATAAAATTCTTGAAGAC R-TTACCAATGCTTAATCA	Kim et al. (2013)

Extraction of genomic DNA was done by TIANamp Bacteria DNA Kit (Tiangen Biotech, China), and it was stored at −20 ˚C until use. The DNA concentration was determined with a spectrophotometer (NanoDrop1000, Thermo Scientific, USA) at a wavelength of 260 nm, and 100 ng/μL concentration was adjusted with sterile deionized water. Finally, it was utilized as DNA template.

The PCR amplifications were done using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR cycling conditions for *groEL* genes of *V. parahaemolyticus* included primary denaturation at 95 ˚C for 10 min, followed by secondary denaturation at 95 ˚C for 15 s, annealing and extension at 69 ˚C for 1 min; for *tetA, tetB, tetC*, and bla_{TEM} genes of *V. parahaemolyticus*, primary denaturation at 95 °C for 4 min, followed by secondary denaturation at 95 °C for 30 s, annealing and extension at 55 °C for 1 min. At the time of annealing step of each cycle, we recorded the increase in fluorescence. The real-time PCR assays of *groEL, tetA, tetB, tetC,* and bla_{TEM} genes were run separately, and increases in fluorescence after 40 cycles were regarded as negative. As a negative control, a PCR mixture without DNA was taken into consideration.

A 25 μL of PCR mixture comprising 12.5 μL of TB Green master mix (Takara Bio, Japan), 5 μL of genomic DNA, 1 μL of each of the designated forward and reverse primers (Macrogen, Inc. South Korea), and 5.5 μL of nuclease-free water were utilized for amplification.

Antibiotic susceptibility test of *V. parahaemolyticus*. The antibiotic susceptibility test (AST) of *V. parahaemolyticus* was performed by employing the disk diffusion method (Bauer et al., 1966). A total of 7 different antimicrobial classes including 12 commercially available antibiotic discs where most of these antibiotics are used in Bangladesh for fighting bacterial infections in aquaculture industry like shrimp hatcheries were selected (Kawsar et al., 2019; Rahman, 2014; Chowdhury et al., 2012; Uddin & Kader, 2006). Aminoglycosides (Gentamicin 10 μg-CN), cephalosporins (Ceftriaxone 30 μg-CRO, Cephradine 30 μg-CE), fluoroquinolones (Ciprofloxacin 5 μg-CIP), penicillins (Amoxicillin 10 μg-AX, Penicillin G 10U-P, Cloxacillin 10 μg-CX, Oxacillin 10 μg-OX, Ampicillin 10 μg-AM), tetracyclines (Tetracycline 10 μg-TE) sulfonamides (Trimethoprim/sulphamethoxa zole 25 μg-SXT), and glycopeptide (Vancomycin 30 μg-VA) were identified as commonly used antibiotics. All the antibiotic discs were purchased from Bioanalyse, Turkey. Following incubation for 18–24 h at 37 °C on TCBS agar plates, preparation of a direct colony suspension was done by taking 2–3 bacterial colonies in sterile 0.85% sodium chloride (NaCl) solution to achieve the final bacterial concentration of 1.5 x 10^8 CFU/mL, that was equivalent to 0.5 McFarland standard. The inoculum was evenly spread on the Mueller-Hinton agar plates (MHA; Liofilchem, Italy) by sterile cotton swabs, and then, the plates were dried for 5–10 min. Antibiotic discs were spotted on the MHA plates by using sterile forceps. The MHA plates were then incubated at 37 °C for 18–24 h.

AST interpretive criteria. A millimeter scale was used to measure the diameter of zone of inhibition around the antibiotic disc. The value of the diameter of each zone of inhibition was applied to distinguish each isolate of *V. parahaemolyticus* as susceptible (S), intermediate (I), or resistant (R) as stated in the rules of Clinical and Laboratory Standards Institute (CLSI, 2018). Isolates of *V. parahaemolyticus* were thought to be as multidrug resistant (MDR) that revealed resistance to not less than three antimicrobial categories (Sweeney et al., 2018). The limit of antibiotic pollution in the *V. parahaemolyticus* isolates was also identified through multiple antibiotic resistance (MAR) index analysis (Osundiya et al., 2013). To calculate the MAR index, the formula, a/b, where "a" is the number of antibiotics to which the selective isolate showed resistance and "b" is the total number of antibiotics used in this study was applied (Krumperman, 1983). While the MAR index was <0.2, then isolates were thought to be from minimal-risk sources of antibiotic pollution, and while the MAR index was >0.2, then, isolates were considered from higher-risk sources of antibiotic contamination (Ballah et al., 2022).

Electro thermal heater digestion for detection of heavy metals. A total of 10 mL HNO_3 and 5 mL $HClO_4$ solution were used to treat the samples. After treatment, an electro-thermal heater (Model-VELP) was used to digest exactly 1 g from each sample at 80 °C for 30 min. After digestion, the samples were cooled and transferred into clean volumetric flasks. To make each solution exactly 100 mL, double distilled water was added. Finally, prior to keeping in sealed and labeled plastic bottles, filtration of the solutions was done by Whatman Filter paper No. 42.

Blank preparation for detection of heavy metals. To confirm that impurities (if any) from the chemicals did not bias the values, a blank containing the same digestion inputs except the sample was prepared using a standard procedure (Shovon et al., 2017). To get the true value, subtraction of the blank value found through the analysis by AAS was done from each of the sample values.

Sample analysis for detection of heavy metals. To determine the concentration of heavy metals, a flame atomic absorption spectrophotometer (Model Shimadzu AA-7000) was used, where as fuel and oxidizer, acetylene gas, and air were used, respectively. The air acetylene flame was used for aspiration of the digested samples. With the support of calibration curves relying on Beer Lambert's law, the concentrations of heavy metals were determined (Skoog et al., 2021). Using standard solutions as the manufacturer's protocol, calibrations by consecutive dilution were achieved. Determination was based on average values of triplicates for each sample. Absorption wavelengths of 228.0 nm, 217.0 nm, 213.9 nm, 248.3 nm, and 357.9 nm were maintained for determination of concentration of Cd, Pb, Zn, Fe, and Cr, respectively. Spectrophotometer's detection limit is 0.01 mg/kg, and the concentrations below the limit were termed as BDL (below the detectable limit).

Statistical analysis. Entry of all the collected data in the Excel 365 (Microsoft/Office 365, Redmond, DC, USA) spreadsheet was done, and then, the data were inspected for any inconsistency and error before being categorized, and analyzed to ensure that they maintained their accuracy. For heavy metals analysis, all the data were then processed to produce a graphical and tabular presentation comparing with maximum recommended limits (FAO/WHO, 1984). On the other hand, for antibiotic resistance genes, the data were shifted from Excel 365 to the statistical package for social sciences (IBM SPSS 26.0, Chicago, IL, USA) and GraphPad Prism 9.5.1 (GraphPad Software, Inc.) for other statistical analysis. Calculation of the binomial 95% confidence intervals (CI) was done using SPSS. Moreover, a chi-square (χ^2) test was executed to figure out the variations of *V. parahaemolyticus* among various sorts of collected samples by using SPSS. The statistically significant p value was set at ≤ 0.05. A heatmap was created by GraphPad Prism to display the antibiotic resistance profiles of *V. parahaemolyticus* isolates.

Human health risk assessment for heavy metals. To assess the potential health risk, a scientific formula (Eq. (1)) established by USEPA was used to calculate the THQ for each heavy metal (USEPA, 2010). The overall HI and TR posed by the determined heavy metals were also calculated by the scientific formulas (Eqs. (2) and (3)) (USEPA, 2010; Bonsignore et al., 2018).

$$THQ = \frac{E_D \times F_{IR} \times E_F \times C_i}{R_{FD} \times W_{AB} \times T_A} \times 10^{-3} \quad (1)$$

where

E_D = Exposure duration (Average life span, 72.32 years).
F_{IR} = Daily ingestion rate (2.43 g/person/day, determined from an online-based survey with 5 thousand respondents throughout the country).
E_F = Exposure frequency (365 days/year).
C_i = Concentration of respective heavy metal (mg/kg).
R_{FD} = The reference oral dose in mg/kg/day (0.001 for Cd, 0.004 for Pb, 1.5 for Cr, 0.3 for Zn, 0.007 for Fe according to USEPA, 2010).
W_{AB} = Average body weight for an adult consumer (54.6 kg for Bangladesh, according to the online-based survey).
T_A = Average exposure time, calculated as $E_D \times E_F$.

The overall hazard index (HI) was calculated using the following formula (Eq. (2)) according to USEPA (2010).

$$HI = THQ_{Fe} + THQ_{Zn} + THQ_{Cd} + THQ_{Pb} + THQ_{Cr} \quad (2)$$

Among the analyzed heavy metals, Cd, Cr, Pb were considered as potent carcinogens. TR posed by the determined heavy metals was calculated according to following formula (Eq. (3)) (Bonsignore et al., 2018):

$$TR = \frac{E_D \times F_{IR} \times E_F \times C_i \times C_{SF}}{W_{AB} \times T_A} \times 10^{-3} \quad (3)$$

The values of cancer slope factors (C_{SF}) were adopted from USEPA (2010) (for Cd (6.3 mg/kg/day) and Pb (0.0085 mg/kg/day)) and Zeng et al. (2015) (for Cr (0.5 mg/kg/day)).

Ethical statement. The Ethical Committee of Bangladesh Agricultural University approved the study protocol (ESRC/47/2024/BAURES/24.08.2024).

Results

Percentages of shrimp, water, and sediment samples positive for *V. parahaemolyticus*. Thirty-nine isolates were suspected as *V. parahaemolyticus* from 130 samples, and the percentages of shrimp, water, and sediment samples positive for *V. parahaemolyticus* are presented in Table 3. The prevalence percentage of *V. parahaemolyticus* in different samples was recorded as 30% (39/130, 95% CI: 22.3–38.7%), and their relationship was insignificant (Table 3). In this case, the prevalence rate was highest in sediment (33.33%) followed by shrimp (30.26%), and water (25.93%) (Table 3). Moreover, samples from 21 ponds were recorded as contaminated with *V. parahaemolyticus* and 77.78% (21/27, 95% CI: 57.7–91.4%) ponds were marked as contaminated with bacteria.

Percentages of samples from different sampling sites positive for *V. parahaemolyticus* in TCBS agar. All the suspected *V. parahaemolyticus* isolates were detected by color, shape, and size of the colonies on TCBS agar, and by Gram's staining method. All the isolates produced green, round colonies that were 2–5 mm in diameter. In Gram's staining, they were recorded as Gram-negative, asporogenous rods which were straight or had a single, rigid curve. Percentages of samples from different sampling sites positive for *V. parahaemolyticus* in TCBS agar are presented in Table 4 where their relationship was insignificant. In this case, the prevalence rate was highest in Debhata (35%, 95% CI: 15.4–59.2%) followed by Dumuria (31.7%, 95% CI: 18.1–48.1%), Kaliganj (28.84%, 95% CI: 17.1–43.1%), and Satkhira Sadar (23.52%, 95% CI: 6.8–49.9%).

Biochemical characterization of *V. parahaemolyticus*. All the isolates that were thought to be positive for *V. parahaemolyticus* in cultural and morphological characterization techniques were positive to catalase and oxidase tests. Not a single suspected *V. parahaemolyticus* isolate was positive to MR and urease tests. All the isolates were positive to the indole production test and nitrate reduction test. In sugar fermentation test, they fermented glucose, sucrose, mannose, maltose, and mannitol but were late lactose-fermenters.

Molecular detection of *V. parahaemolyticus*. For species identification, 27 isolates were selected on the basis of their DNA concentration. Twenty of the 27 suspected *V. parahaemolyticus* isolates (74.07%) were revealed as positive for the *groEL* gene through showing distinct amplification curves and C_t (threshold cycle) values before the completion of 40 cycles using species-specific primers (Fig. S1). Among 20 isolates of *V. parahaemolyticus*, 12 isolates (60%) showed positive result to *tetA* gene (Fig. S2), 13 (65%) were positive to *tetB* gene (Fig. S3), 12 (60%) were positive to *tetC* gene (Fig. S4), and 1 (5%) was positive to bla_{TEM} gene (Fig. S5). In all these cases, distinct amplification curves and C_t values were recorded.

Antibiogram profiles of *V. parahaemolyticus*. All the 39 isolates both confirmed and suspected were tested for investigating the antibiotic resistance patterns of the circulating *V. parahaemolyticus* and other bacteria (if present) in shrimp and their environments. As a result, farm owners will be able to understand the overall antibiotic resistance patterns of *V. parahaemolyticus* and other bacteria in his/her farms. Most of the *V. parahaemolyticus* isolates were phenotypically resistant to tetracycline and ampicillin (56.4%; 95% CI:39.6–72.2%), followed by vancomycin (53.8%; 95% CI:37.2–69.9%), cloxacillin (51.2%; 95% CI:34.8–67.6%), oxacillin (46.1%; 95% CI:30.1–62.8%), trimethoprim/sulphamethoxazole (33.3%; 95% CI:19.1–50.2%), penicillin G (23.07%; 95% CI:11.1–39.3%), cephradine (17.9%; 95% CI:7.5–33.5%), ceftriaxone (5.1%; 95% CI:0.6–17.3%), gentamicin, ciprofloxacin, and amoxicillin (2.6%; 95% CI:0.1–13.5%) (Fig. 2).

Bivariate analysis showed a significant positive correlation between the resistance patterns of oxacillin and ceftriaxone (Spearman coefficient, $\rho = 0.363$), ampicillin and tetracycline (Spearman coefficient, $\rho = 0.366$) (Table 5). Furthermore, a significant negative correlation was recorded between amoxicillin and tetracycline (Spearman coefficient, $\rho = -0.336$) (Table 5). Also, the correlation between every two antibiotics was recorded (Table 5).

MDR and MAR profiles of *V. parahaemolyticus*. Twenty of 39 *V. parahaemolyticus* isolates (51.28%) were MDR in nature. All the MDR isolates had >0.2 MAR index (Table 6). The range of MAR indices of the isolates was from 0.08 to 0.6 (Table 6). Moreover, *V. para-*

Table 3
Percentages of samples (shrimp, water, and sediment) positive for *V. parahaemolyticus*

Samples positive for *V. parahaemolyticus*				
Shrimp (n = 76) *N* (%)[P] (CI)[Q]	Water (n = 27) *N* (%)[P] (CI)[Q]	Sediment (n = 27) *N* (%)[P] (CI)[Q]	*p* value	Total (n = 130) *N* (%)[P] (CI)[Q]
23 (30.26) (20.2–41.9)	7 (25.93) (11.1–46.3)	9 (33.33) (16.5–54.0)	0.836	39 (30) (22.3–38.7)

Values differ significantly ($p \le 0.05$) within the variable under assessment, CI = confidence interval, P = number and percentage of positive isolates, Q = 95% confidence interval.

Table 4
Percentages of samples (shrimp, water, and sediment) from different sampling sites positive for *V. parahaemolyticus* in TCBS agar

Samples from different sampling sites positive for *V. parahaemolyticus* in TCBS agar					
Satkhira Sadar (n = 17) *N* (%)[P] (CI)[Q]	Dumuria (n = 41) *N* (%)[P] (CI)[Q]	Debhata (n = 20) *N* (%)[P] (CI)[Q]	Kaliganj (n = 52) *N* (%)[P] (CI)[Q]	*p* value	Total (n = 130) *N* (%)[P] (CI)[Q]
4 (23.52) (6.8–49.9)	13 (31.7) (18.1–48.1)	7 (35) (15.4–59.2)	15 (28.84) (17.1–43.1)	0.459	39 (30) (22.3–38.7)

TCBS = Thiosulfate-Citrate-Bile Salts-Sucrose, values differ significantly ($p \le 0.05$) within the variable under assessment, CI = confidence interval, P = number and percentage of positive isolates, Q = 95% confidence interval.

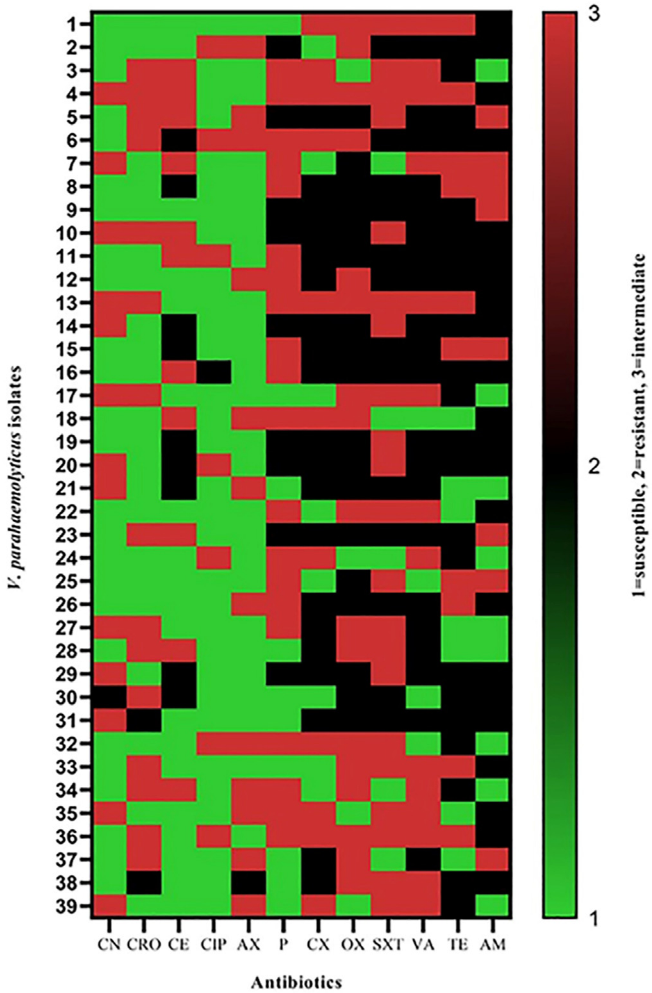

Figure 2. Heatmap representing the antimicrobial resistance patterns of each *V. parahaemolyticus* isolated from shrimp and shrimp environments; AM = ampicillin; AX = amoxicillin; CE = cephradine; CIP = ciprofloxacin; CN = gentamicin; CRO = ceftriaxone; CX = cloxacillin; OX = oxacillin; P = penicillin G; SXT = trimethoprim/sulphamethoxazole; TE = tetracycline; VA = vancomycin.

haemolyticus isolates showed 18 different antimicrobial-resistant patterns (Table 6).

Concentrations of heavy metals. The concentrations of all the heavy metals observed from analyzed shrimp samples are presented in Table 7.

Fe concentrations. The highest average concentration level of Fe (299.965 ± 15.479 mg/kg) was recorded from the shrimps of Satkhira Sadar whereas the lowest average (187.0583 ± 24.3603 mg/kg) was from the shrimps of Dumuria. However, the average concentrations of Fe determined from all sampling sites crossed the maximum recommended limit (FAO/WHO, 1984).

Zn concentrations. The highest average level of Zn (82.986 ± 4.769 mg/kg) was observed in the shrimps of Satkhira Sadar, while the shrimps of Dumuria revealed the lowest average concentration (70.319 ± 5.1544 mg/kg). However, average concentrations of Zn recorded from shrimps of different subdistricts were lower than the recommendation (FAO/WHO, 1984).

Cr concentrations. Shrimps of Satkhira Sadar and Kaliganj were afflicted with Cr with an average concentration of 0.0865 (±0.0092) and 0.0871 (±0.0769) mg/kg, respectively, where both values crossed the recommendation. Average concentrations of chromium in the other subdistricts were lower than the recommended value.

Pb concentrations. The maximum average Pb concentration (0.3513 ± 0.0264 mg/kg) was reported from Dumuria, while the minimum (0.0853 ± 0.0140 mg/kg) from Kaliganj. However, concentration values recorded from all sampling sites were far below than the maximum recommended value (FAO/WHO, 1984).

Cd concentrations. The highest average Cd concentration (0.0827 ± 0.0095 mg/kg) was recorded from the shrimps of Debhata, while the lowest (0.0456 ± 0.010 mg/kg) from the shrimps of Dumuria. However, no determined concentration of heavy metals crossed the maximum recommended limits defined by FAO and WHO (FAO/WHO, 1984).

Human health risk assessment. Though the determined concentrations of Fe and Cr (in Satkhira Sadar and Kaliganj) crossed the recommended values, THQ values solely for Fe were higher than 1 in all subdistricts (Table 8). This made the HI elevated over 1 in all sites (Table 8). From spatial consideration, shrimps of Kaliganj possessed the highest level of noncarcinogenic health risk, whereas Satkhira Sadar did the lowest (Table 8). No determined TR value, nor their additive effect (TRt) exceeded the recommended value ($<10^{-4}$)

Table 5
Assessment of the significant relation between any of the two antibiotics resistant to *V. parahaemolyticus* by the calculation of Spearman coefficient

		CN	CRO	CE	CIP	AX	P	CX	OX	SXT	VA	TE	AM
CN	ρ	1											
CRO	ρ	0.065	1										
CE	ρ	0.003	0.177	1									
CIP	ρ	−0.239	−0.167	−0.043	1								
AX	ρ	−0.169	−0.099	−0.143	0.065	1							
P	ρ	−0.211	−0.197	0.144	0.285	0.017	1						
CX	ρ	0.023	−0.014	−0.029	0.229	0.164	0.248	1					
OX	ρ	−0.218	0.363*	−0.212	0.053	0.163	−0.025	−0.095	1				
SXT	ρ	0.279	0.172	−0.190	−0.131	−0.269	−0.184	0.163	0.077	1			
VA	ρ	0.155	0.209	−0.183	−0.107	−0.133	0.013	0.153	0.070	0.234	1		
TE	ρ	−0.087	−0.020	−0.066	0.028	−0.336*	0.206	−0.004	−0.022	0.074	0.219	1	
AM	ρ	−0.217	−0.130	0.089	−0.175	−0.141	0.072	−0.227	−0.114	−0.228	−0.251	0.366*	1

AM = ampicillin; AX = amoxicillin; CE = cephradine; CIP = ciprofloxacin; CN = gentamicin; CRO = ceftriaxone; CX = cloxacillin; P = penicillin G; OX = oxacillin; SXT = trimethoprim/sulphamethoxazole; TE = tetracycline; VA = vancomycin; ρ = spearman correlation coefficient.

* Correlation is significant at the 0.05 level (2-tailed).

Table 6
Multidrug resistance (MDR) and multiple antibiotic resistance (MAR) index of *V. parahaemolyticus* isolated from shrimp and their environments

Pattern No.	Antibiotic resistance patterns	No. of antibiotics (classes)	No. of isolates	Overall No. of MDR isolates (%)	MAR index
1	AM, TE, VA, SXT, OX, CX, CIP	7 (5)	1	20/39 (51.28)	0.6
2	AM, TE, VA, OX, CX, P, CE	7 (4)	4		0.6
3	AM, TE, VA, SXT, OX, CX, CRO	7 (5)	1		0.6
4	AM, TE, VA, SXT, OX, CX	6 (4)	1		0.5
5	AM, TE, VA, OX, CX, P	6 (4)	1		0.5
6	TE, VA, SXT, OX, CX, P	6 (4)	2		0.5
7	AM, TE, SXT, OX, CE, CN	6 (5)	1		0.5
8	AM, TE, VA, SXT, P	5 (4)	1		0.4
9	AM, TE, VA, SXT, CE	5 (5)	1		0.4
10	AM, TE, CX, AX, CRO	5 (3)	1		0.4
11	TE, VA, OX, CX, P	5 (3)	1		0.4
12	AM, TE, VA, SXT, CX	5 (4)	1		0.4
13	VA, SXT, OX, CX, CE	5 (4)	3		0.4
14	AM, VA, SXT, OX, CX	5 (3)	1		0.4
15	VA, CX	2 (2)	3		0.1
16	AM	1 (1)	8		0.08
17	TE	1 (1)	6		0.08
18	OX	1 (1)	2		0.08

AM = ampicillin; AX = amoxicillin; CE = cephradine; CIP = ciprofloxacin; CN = gentamicin; CRO = ceftriaxone; CX = cloxacillin; P = penicillin G; OX = oxacillin; SXT = trimethoprim/sulphamethoxazole; TE = tetracycline; VA = vancomycin.

Table 7
Average concentration of heavy metals (Fe, Zn, Cr, Pb, and Cd) in shrimp muscles collected from four selected areas of Khulna and Satkhira districts

Sampling site	Concentrations of heavy metals (mg/kg)				
	Cd	Cr	Pb	Fe	Zn
Satkhira Sadar	0.0695 ± 0.0247	0.0865 ± 0.0092	0.089 ± 0.004	299.965 ± 15.479	82.986 ± 4.769
Dumuria	0.0456 ± 0.010	0.0461 ± 0.0104	0.3513 ± 0.0264	187.0583 ± 24.3603	70.319 ± 5.1544
Debhata	0.0827 ± 0.0095	0.045 ± 0.0096	0.124 ± 0.0572	253.54 ± 58.1176	79.4943 ± 11.9089
Kaliganj	0.0715 ± 0.0155	0.0871 ± 0.0769	0.0853 ± 0.0140	289.6665 ± 22.6973	77.3769 ± 8.2872

Table 8
Values of THQ, HI, and TR for each heavy metal traced from the shrimp muscles

Risk indexes	Satkhira Sadar	Dumuria	Debhata	Kaliganj
THQ				
THQ_{Zn}	0.012311	0.010432	0.011793	0.011479
THQ_{Cd}	2.20938E−05	1.4487E−05	2.62794E−05	2.27296E−05
THQ_{Cr}	2.56648E−06	1.36907E−06	1.33516E−06	2.58503E−06
THQ_{Pb}	0.00099	0.003909	0.00138	0.000949
THQ_{Fe}	**1.907156**	**8.325122**	**11.28392**	**12.89175**
HI	**1.920482**	**8.339478**	**11.29712**	**12.9042**
TR				
TR_{Cd}	1.2311E−05	1.0432E−05	1.1793E−05	1.1479E−05
TR_{Cr}	3.85E−06	2.05E−06	2.00E−06	3.88E−06
TR_{Pb}	3.96E−06	1.56E−05	5.52E−06	3.80E−06
TRt	2.01E−05	2.81E−05	1.93E−05	1.92E−05

THQ = target hazard quotient; HI = hazard index; TR = target cancer risk.
Values exceeded recommendation are indicated as bold.

(Table 8). However, Dumuria presented the highest TR values contributed largely by Cr, whereas Kaliganj did the lowest (Table 8).

Discussion

The present research work explored shrimp and their environments for the detection of antibiotic resistance genes of *V. parahaemolyticus* and performed spectrophotometry of shrimp muscles for heavy metals and their human health risk assessment in Bangladesh.

In this research, phenotypic characterization of the isolates recorded 30% of isolates as *V. parahaemolyticus* from 130 samples (Table 3). The observations of this research work were comparable with the observations of the earlier study (Haifa-Haryani et al., 2022). In Southern Bangladesh, a similar study was carried out where 27% of shrimp samples were recorded as infected with *V. parahaemolyticus* (Siddique et al. 2021). Another study was carried out in Maharashtra, India where 72.5% of isolates confirmed as *V. parahaemolyticus* out of 120 isolates (Kohli et al., 2021). In this research work, 74% of isolates showed a positive result for *groEL* gene (Fig. S1) agreeing with the findings of a similar study in Southern Bangladesh where 15.3% samples were positive for *groEL* gene out of 150 samples (Haque et al., 2023). Some other previous studies reported more or less similar data for *groEL* gene in *V. parahaemolyticus* isolates (Hossain et al., 2013a, 2013b; Siddique et al., 2021). Geographical distributions, environments, test methodologies, sample types, sizes, and collection time might play a significant role in the variations between these findings and our findings. Since the concentration of most *Vibrio* spp. is greatly influenced by the water surface temperature and salinity, vibriosis usually appears during the period of summer and fall when surface waters remain relatively warm (Brumfield et al., 2023). In this research work, sample collection was done in comparatively cold weather. Due to this reason, the prevalence percentages of *V. parahaemolyticus* were not so high.

Antimicrobial resistance (AMR) phenomenon is one of the top global problems, becoming a progressively serious risk to public health (Bag et al., 2022). In this research work, 56.4% of *V. parahaemolyticus* revealed resistance to ampicillin and tetracycline (Fig. 2) that shows compliance with the previous studies (Haifa-Haryani et al., 2022; Siddique et al., 2021). We recorded 23.07% *V. parahaemolyticus* isolates as resistant to penicillin G (Fig. 2). The observations of the present research work were in line with a similar research work conducted in Kaliganj upazilla in Satkhira district where 28.57% *Vibrio* isolates showed resistance to penicillin (Hossain et al., 2012a, 2012b). In the earlier studies conducted in Peninsular Malaysia, 53.84% of isolates were recorded as resistant to Penicillin G (Haifa-Haryani et al., 2022). We recorded 53.8% *V. parahaemolyticus* isolates as resistant to vancomycin (Fig. 2) and it was related to another study (Haifa-Haryani et al., 2022). In the present research work, 2.6% *V. parahaemolyticus* isolates were recorded as resistant to ciprofloxacin, gentamicin, and amoxicillin (Fig. 2). In line with our findings, earlier studies reported that *Vibrio* spp. were highly susceptible to ciprofloxacin, and gentamicin (Haque et al., 2023, Yu et al., 2023). A total of 33.3% isolates of *V. parahaemolyticus* were revealed as resistant to trimethoprim/sulphamethoxazole (Fig. 2). Almost similar level of resistance to trimethoprim/sulphamethoxazole was reported by another study (Yu et al., 2023). The present study showed that 5.1% isolates of *V. parahaemolyticus* were resistant to ceftriaxone (Fig. 2). Another study conducted in Saudi Arabia recorded 97.5% isolates of *V. parahaemolyticus* as sensitive to ceftriaxone (Elhadi et al., 2022). Almost 50% of isolates of *V. parahaemolyticus* were resistant to oxacillin and cloxacillin whereas almost 82% of *V. parahaemolyticus* isolates were susceptible to cephradine (Fig. 2).

A significant positive correlation was recorded between the resistance patterns of oxacillin and ceftriaxone, ampicillin, and tetracycline by bivariate analysis (Table 5). Moreover, amoxicillin and tetracycline revealed a significant negative correlation (Table 5). A similar research work was conducted in the South-Western part of Bangladesh where different antibiotics showed a very high significant positive correlation between their resistance patterns (Haque et al., 2023). In aquaculture systems, the use of antibiotics randomly might be the probable reason of the other significant relations.

AMR paves the way for spreading MDR isolates where they create a significant risk to healthcare systems all around (Sobur et al., 2022). In this research work, 51.28%isolates of the *V. parahaemolyticus* were MDR in nature (Table 6) and this finding showed agreement with the results of the earlier research works (Elhadi et al., 2022). In this study, all the MDR isolates of the *V. parahaemolyticus* had >0.2 MAR index value, that reveals that samples were from a highly risk contaminated area where a number of antibiotics were used (Table 6). Previous studies also showed agreement with the finding of the present study by reporting more than 50% of *Vibrio* isolates with >0.2 MAR index value (Mohamad et al., 2019). Though farmers in this study declared that they were not involved in using any antibiotics on their shrimp farms, the findings were not analogous and revealed that the occurrence of MAR was extreme. Differences in the geographical location of the sample collection areas may influence the variation in the resistance level (Lesley et al., 2011).

This research work revealed the complementary approach of antibiotic susceptibility and PCR assays. Phenotypic analysis like antibiotic susceptibility test by disk diffusion technique often induces false negative interpretations on the basis of only phenotypic characteristics (Galhano et al., 2021). Most of the time the PCR test exhibits the existence of antibiotic resistance genes in many of the phenotypically resistant strains and the absence of resistance genes in some other strains that demonstrate phenotypic resistance (Adesiyan et al., 2022). Therefore, in this study, only the confirmed isolates of *V. parahaemolyticus* by real-time PCR were subjected to the detection of antibiotic resistance genes for better interpretations of the findings. In the present research work, 60% of isolates of *V. parahaemolyticus* were recorded as the carriers of *tetA* and *tetC* genes (Figs. S2 and S4) whereas 65% of isolates were the carriers of *tetB* gene (Fig. S3). A total of 5% *V. parahaemolyticus* isolates were the carriers of bla_{TEM} gene (Fig. S5). A study carried out in the retail shrimps in Malaysia where β-lactam resistance genes usually recorded as plasmid-encoded β-lactamase and tetracycline resistance genes among the isolates of *V. parahaemolyticus* were not detected (Letchumanan et al., 2015). A study carried out in *Escherichia coli* in the Philippines reported 47.66% of isolates as the carrier of bla_{TEM} and 45.33% of isolates as the carrier of *tetA* gene (Salvador-Membreve & Rivera, 2021).

Table 7 shows that the concentrations of Fe in all shrimps were far higher than the recommended limit (FAO/WHO, 1984). The results of the present study also crossed all other findings of 6.570 mg/kg (Mendil et al., 2010) and of 8.819 mg/kg (Minganti et al., 2010) in fishes from Turkey and Italy, respectively. However, the results were almost similar to the findings of another study (Biswas et al., 2021). More than 1of THQ_{Fe} values from the study areas reveal that shrimps were not safe for human consumption (Table 8). The concentrations of Zn (Table 6) were more or less similar to the findings of another study (Biswas et al., 2021), but higher than the findings observed in fishes in another study (Alam et al., 2023) and lower than the concentrations of the south west coast of India (Rejomon et al., 2010) and Iran (Biswas et al., 2012). However, average values of THQ_{Zn} in all sampling sites were within the recommendation, and the shrimps were safe for human consumption (Table 8). The findings of Cr concentrations (Table 7) agree with the findings obtained from a study conducted in the Khulna Division of Bangladesh (Ahmed et al., 2023) but differ from the findings of another study (Alam et al., 2023). The concentrations of Cr in Satkhira Sadar and Kaliganj crossed the maximum recommended values of WHO and FAO (Tables 1 and 7) might be due to the uptake of Cr-rich wastes into shrimp body. Fortunately, values of THQ_{Cr} were very negligible, and confirmed no potent health risk for humans from Cr consideration (Table 8). The concentrations of Pb in Satkhira Sadar, Debhata, and Kaliganj in this study (Table 7) were somewhat close to the results reported by another study (Ahmed et al., 2023). The finding of Pb concentration in Dumuria was 0.354 ± 0.040 in a study carried out in Khulna (Biswas et al., 2021) agreeing with the finding of this study. However, the results of Pb concentrations in this study differ from the findings of another study in fish samples (Alam et al., 2023). The shrimps were safe for human consumption because Pb concentrations were far lower than the recommendation of WHO and FAO, and lower average THQ_{Pb} values in all sampling sites (Table 8). The results of Cd concentrations in all sampling sites in this study (Table 7) agree with the findings of other studies conducted in Khulna, Bagerhat, Satkhira, and Natore of Bangladesh (Ahmed et al., 2023; Alam et al., 2023). However, the finding in shellfish (1.50 mg/kg) from the Buriganga river (Ahmed et al., 2015) differs from our findings. In this study, lower THQ_{Cd} values (Table 8), and lower average Cd concentrations in all shrimps deny the health risks posed by high doses of Cd exposure.

Conclusions

The findings observed in this research work report the comprehensive information about the occurrence of *V. parahaemolyticus* in shrimp and their environments, the detection of their antibiotic resistance genes, and concentration of heavy metals in shrimp muscles. The present research work also determined the potential human health risk of the targeted heavy metals. Based on observations, our study suggests that for sustainable shrimp production, extensive surveillance, and monitoring of a large number of shrimp farms and samples should be encouraged for effective antibiotic management and for decreasing heavy metals' concentrations. Farm owners should be aware of the potential risks of antibiotic-resistant *Vibrio* contamination and take appropriate measures at the time of culture, harvest, and processing of shrimp. Moreover, it is necessary to identify the possible risk factors which help in the outbreak of vibriosis in shrimp industry and increase the load of heavy metals that can cause possible health hazards to humans. Another crucial thing is the circulation of multidrug-resistant *V. parahaemolyticus* in shrimps and their environments that results in a possible risk to consumer health through causing acute gastroenteritis, wound infections, and sepsis. So, continuous monitoring of antibiotic resistance of *V. parahaemolyticus* is essential for the most effective treatment of patients suffering from these anomalies and to ensure the safety of seafood.

CRediT authorship contribution statement

M. Sohidullah: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Md. Hamidur Rahman:** Writing – review & editing, Resources, Methodology. **Abu Sayeed:** Writing – review & editing, Visualization, Methodology. **Sadia Rahman:** Writing – review & editing, Visualization, Data curation. **Linta Yesmin:** Writing – review & editing, Visualization. **Md. Imran Chowdhury:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis. **Md. Jannat Hossain:** Writing – review & editing, Software, Methodology. **Muhammad Ashiqul Alam:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Conceptualization. **Md. Salauddin:** Writing – review & editing, Software, Methodology. **Md. Habibur Rahman:** Writing – review & editing, Resources, Methodology. **Md. Tazinur Rahman:** Writing – review & editing, Resources, Methodology. **Sayeed Khaled Sabbir:** Writing – review & editing, Software.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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