Prevalence, multidrug resistance, and biofilm formation of *Vibrio parahaemolyticus* isolated from fish mariculture environments in Cat Ba Island, Vietnam

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**ABSTRACT**

Objectives: *Vibrio parahaemolyticus* is a major foodborne pathogen in aquatic animals and a threat to human health worldwide. This study investigated the prevalence, antimicrobial resistance, antimicrobial resistance genes (ARGs), and biofilm formation of *V. parahaemolyticus* strains isolated from fish mariculture environments in Cat Ba Island, Vietnam.

Methods: In total, 150 rearing water samples were collected from 10 fish mariculture farms in winter and summer. A polymerase chain reaction assay was used to identify *V. parahaemolyticus*, its virulence factors, and ARGs. The antimicrobial resistance patterns and biofilm formation ability of *V. parahaemolyticus* strains were investigated using the disk diffusion test and a microtiter plate-based crystal violet method, respectively.

Results: Thirty-seven *V. parahaemolyticus* isolates were recovered from 150 samples. The frequencies of the *tdh* and *trh* genes among *V. parahaemolyticus* isolates were 8.1% and 21.6%, respectively. More than 90% of isolates were susceptible to ceftazidime, cefotaxime, and chloramphenicol, but over 72% were resistant to ampicillin, tetracycline, and erythromycin. Furthermore, 67.57% of isolates exhibited multidrug resistance. The presence of ARGs related to gentamicin (*aac(3)-IV*), tetracycline (*tetA*) and ciprofloxacin (*qnrA*) in *V. parahaemolyticus* isolates was identified. Conversely, no ARGs related to ampicillin or erythromycin resistance were detected. Biofilm formation capacity was detected in significantly more multidrug-resistant isolates (64.9%) than non-multidrug-resistant isolates (18.9%).

Conclusion: Mariculture environments are a potential source of antibiotic-resistant *V. parahaemolyticus* and a hotspot for virulence genes and ARGs diffusing to aquatic environments. Thus, the prevention of antibiotic-resistant foodborne vibriosis in aquatic animals and humans requires continuous monitoring.

Keywords: Antimicrobial resistance; Biofilm; Multidrug resistance; *Vibrio parahaemolyticus*; Virulence factor
Introduction

Aquaculture environments are considered to be reservoirs of various biological pollutants, including infectious agents and antimicrobial resistance genes (ARGs) [1]. ARGs, which are contaminants of emerging concern [2], encode antimicrobial resistance and play an essential role in the process of resistance prevalence and proliferation [3]. In the environment, ARGs can be transferred from one microorganism to another, including from non-pathogenic to pathogenic species, through horizontal gene transfer. Pathogenic microorganisms that carry ARGs within aquaculture settings represent one of the most significant global health hazards, with millions of individuals succumbing to foodborne and waterborne diseases annually [4].

*Vibrio parahaemolyticus* is a halophilic marine bacterium that belongs to the family Vibrionaceae and is widely distributed in brackish water and marine environments [5]. This bacterium is a facultative human pathogen and is responsible for approximately 25% of all foodborne diseases closely associated with the consumption of raw seafood [6]. The ingestion of contaminated raw fish and shellfish, or seafood that has not been sufficiently heat-treated, can lead to symptoms such as diarrhea, abdominal pain, vomiting, chills, and low-grade fever in humans [7]. The significance of infections caused by this pathogen is increasing in public health due to a steady rise in its incidence worldwide over recent decades. *V. parahaemolyticus* is considered an emerging pathogen with a global distribution. Its infectious capability is mediated by various virulence factors, including hemolysin, urease, 2 type III secretion systems, and 2 type VI secretion systems, as well as the formation of persister cells [8]. To survive during infections and in seafood, *V. parahaemolyticus* cells may also form biofilms, which shield the cells from host defenses and external factors, including antibiotics, thereby contributing to antimicrobial resistance.

Antibiotics are widely used in aquaculture for both disease prevention and the treatment of infections potentially caused by *V. parahaemolyticus* [4]. However, the long-term use of antibiotics, particularly at incorrect dosages, can lead to the development of antimicrobial resistance in marine bacteria, including *V. parahaemolyticus* [4]. Moreover, antibiotics present in aquatic products and seafood can be ingested by animals and humans, potentially leading to the development of resistance in microorganisms that cause diseases in both animals and humans. This resistance can diminish the effectiveness of these antibiotics in treating infections.

To date, hundreds of *V. parahaemolyticus* strains have been isolated from seafood and shrimp farming environments in Vietnam [9–11]. However, no previous study has investigated the molecular resistance mechanisms and biofilm formation of *V. parahaemolyticus* in marine fish environments in Vietnam. Cat Ba Island is a significant marine fish production area and a well-known tourist destination in the northeast of Vietnam. To mitigate outbreaks caused by the consumption of raw or undercooked fish contaminated with *V. parahaemolyticus*, and to prevent potential risks to human health, it is necessary to monitor the prevalence of *V. parahaemolyticus* in fish mariculture rearing water. Additionally, identifying antimicrobial resistance and ARGs, virulence factor genes, and biofilm formation is of paramount importance. The aim of this study was to improve our understanding of the current state of antimicrobial resistance in the marine environment and the risks posed by the virulence factor genes and ARGs of *V. parahaemolyticus*, which can cause foodborne diseases in aquatic animals and humans.

Materials and Methods

Sampling

The seawater samples were collected following the method described by Zhao et al. [12], with slight modifications. In brief, water from the fish mariculture environment was collected from a depth of 10 to 20 cm below the seawater surface. A total of 150 samples were collected from 10 grouper mariculture farms in Lan Ha Bay (Cat Ba Island), Hai Phong (Figure S1 and Table S1), during the survey period between February 2021 and August 2021.

HIGHLIGHTS

• This is the first study on *Vibrio parahaemolyticus* isolated from fish mariculture in Vietnam.
• 8.1% and 21.6% of isolates harbored the *tdh* and *trh* genes.
• A high percentage of isolates exhibited resistance to ampicillin (86.5%), erythromycin (75.7%), and tetracycline (73.0%), and more than 67% of isolates were multidrug-resistant.
• A close correlation between the resistance phenotypes of *V. parahaemolyticus* and genotype was identified for ciprofloxacin and tetracycline.
• Biofilm formation ability was detected in more multidrug-resistant isolates (64.9%) than non-multidrug-resistant isolates (18.9%).
**Bacteria Isolation**

*V. parahaemolyticus* was isolated as described by Mok et al. [13]. Briefly, 5 mL of seawater was added to a flask containing 95 mL of peptone water with 1.5% NaCl and then incubated at 28 °C for 24 hours. Subsequently, 100 µL aliquots were spread on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Merck) and incubated for 48 hours at 28 °C. Colonies that grew on TCBS were characterized by their color, shape, and size. The blue colonies were subsequently inoculated onto CHROMagar medium (Titan). Purple presumptive colonies were further confirmed through biochemical and polymerase chain reaction (PCR) assays.

**Genomic DNA Extraction and Identification of V. parahaemolyticus**

Colonies of each suspected isolate were cultured in TSB medium (Merck) supplemented with 1.5% NaCl at 28 °C. After 24 hours of incubation, 1.5 mL of the bacterial culture was harvested by centrifugation at 13,000 rpm for 5 minutes. The total genomic DNA from each suspected isolate was then extracted using the Favorgen Tissue Genomic DNA Extraction Mini Kit (Favorgen), following the manufacturer’s instructions. *V. parahaemolyticus* was identified by a PCR assay using *taxR*-specific primers [14]. The genomic DNA of *V. parahaemolyticus* from the collection of the Department of Fisheries, University of Agriculture and Forestry, Hue University was used as a positive control, while nuclease-free water was used as a negative control.

The presence of virulence genes was identified using the primers listed in Table S2. PCR reactions were performed in PTC200 thermal cyclers (Marshall Scientific). Each 25-µL reaction mixture contained 12.5 µL of MyTaq PCR master mix (Bioline), 1 µL of DNA template (50 ng/µL), 1 µL of each primer (10 pmol), and 9.5 µL of distilled water. The PCR program was as follows: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 30 seconds, an annealing temperature of 50 to 60 °C for 30 seconds (varying based on the primer used), and extension at 72 °C for 1 minute, with a final elongation step at 72 °C for 5 minutes. PCR products were visualized by agarose gel electrophoresis using Safe DNA stain (AAT Bioquest).

**Antimicrobial Susceptibility Profiling and PCR Detection of ARGs**

The antimicrobial susceptibility of each *V. parahaemolyticus* isolate was assessed using the disc diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) M45 guidelines [15]. Initially, colonies from each isolate were suspended in 1% NaCl and adjusted to match the 0.5 McFarland standard. This suspension was then spread onto Mueller-Hinton agar plates (HiMedia). Subsequently, discs impregnated with 9 antimicrobial agents (Mast Diagnostics)—ampicillin (10 µg), ciprofloxacin (5 µg), kanamycin (30 µg), gentamicin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), ceftazidime (30 µg), and ceftiofur (30 µg)—were placed equidistantly on the surface of the agar. The plates were then incubated at 28 °C for 18 to 24 hours. *Escherichia coli* ATCC 25922 served as the quality control strain. The resistance profile of *V. parahaemolyticus* was determined by measuring the zones of inhibition, in accordance with the CLSI M45 guidelines for *Vibrio* spp. and the CLSI M100 guidelines [15,16]. The multiple antibiotic resistance (MAR) index was calculated using the formula a/b, where “a” is the number of antibiotics to which resistance was detected in a single isolate, and “b” represents the total number of antibiotics tested [17].

Concurrent with antimicrobial resistance testing, genotype screening was performed using PCR assays to detect the presence of ARGs for ampicillin, gentamicin, ciprofloxacin, erythromycin, and tetracycline. The primers for the targeted ARGs and the PCR conditions are detailed in Table S2.

**Biofilm Formation Assay**

The biofilm production capability of *V. parahaemolyticus* isolates was assessed using a previously described method [18], with some modifications. In brief, overnight cultures of *V. parahaemolyticus* grown in TSB with 1.5% (w/v) NaCl at 30 °C were diluted to an optical density at 600 nm (OD600) of 0.1. Then, 150 µL of the diluted cell suspension was aliquoted in triplicate into 96-well plates (SPL Life Science). After 24 hours of incubation, non-adherent cells and culture medium were removed, and the attached cells were washed 3 times with a 2 mM CaCl₂/MgCl₂ buffer. The cells were then stained with 0.01% crystal violet (Merck) for 15 minutes. Subsequently, the plates were washed 3 more times with the 2 mM CaCl₂/MgCl₂ buffer. To facilitate the measurement of absorbance, 70% ethanol was added to each well before the contents were transferred to a new 96-well plate. Absorbance was measured at a wavelength of 540 nm. The criteria for interpreting the biofilm formation capacity of *V. parahaemolyticus* were as follows, based on a previous report [19]. Briefly, OD > 4×Odc means strong biofilm formation; 2×Odc < OD ≤ 4×Odc, moderate biofilm formation; ODc < OD ≤ 2×Odc, weak biofilm formation; and negative biofilm formation when OD < Odc. Odc refers to the control measurement, which was performed in a microtiter plate without cells.

**Statistical Analysis**

The statistical analysis was carried out using IBM SPSS ver.
20.0 (IBM Corp.). The significance of differences in biofilm formation, the prevalence of \emph{V. parahaemolyticus}, and the distribution of ARGs was examined using the chi-square test. A \( p \)-value \( \leq 0.05 \) was considered as significant.

**Results**

**Prevalence of \emph{V. parahaemolyticus} in Fish Farming Water**

The distribution of \emph{V. parahaemolyticus} in farming water is presented in Table 1. In total, 51 suspected \emph{V. parahaemolyticus} isolates were obtained from a total of 150 samples, using TCBS agar and CHROMagar. These isolates were further verified by a PCR assay (Figure 1A) with \emph{toxR} primers, confirming that 37 of them were \emph{V. parahaemolyticus}. Of these, 11 isolates were collected from 76 samples obtained in the winter, while 26 were identified from 74 samples obtained in the summer. Consequently, the isolation rate of \emph{V. parahaemolyticus} was significantly higher in the summer (35.1%) than in the winter (14.5%) \(( p < 0.05 \), chi-square test).

**Detection of Virulence-Related Genes**

The presence of the \emph{tdh} and \emph{trh} genes in all 37 \emph{V. parahaemolyticus} isolates was examined. The results, as shown in Figure 1 and Table 2, indicated that 3 (8.1%) isolates were positive for the \emph{tdh} gene and 8 (21.6%) isolates were positive for the \emph{trh} gene. However, none of the isolates carried both the \emph{tdh} and \emph{trh} genes. Additionally, biofilm-associated genes were highly prevalent, with 83.8% of isolates harboring the \emph{VP0952} gene, and the \emph{VP0950} and \emph{VP0962} genes were present in 81.08% of isolates.

**Antimicrobial Resistance Pattern of the \emph{V. parahaemolyticus} Isolates**

The antibiotic resistance profile of 37 isolates against 9 antibiotics is presented in Table 3. None of the 37 isolates exhibited resistance to ceftazidime, cefotaxime, or chloramphenicol. A lower resistance rate was observed for ciprofloxacin (18.9%, 7/37), kanamycin (13.5%, 5/37), and

![Figure 1](https://doi.org/10.24171/j.phrp.2023.0181)
Table 2. The distribution of virulence genes, multidrug resistance profile, and biofilm formation of *Vibrio parahaemolyticus* isolates

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>MARI</th>
<th>Resistance phenotype</th>
<th>Biofilm formation</th>
<th>toxR</th>
<th>tdh</th>
<th>thr</th>
<th>VP0950</th>
<th>VP0952</th>
<th>VP0962</th>
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AMP, ampicillin; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; TCN, tetracycline; ERY, erythromycin; +, positive; −, negative.

Table 3. Antimicrobial resistance pattern of *Vibrio parahaemolyticus* isolates

<table>
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<tr>
<th>Antimicrobial agents</th>
<th>Susceptible (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
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<tr>
<td>Ampicillin</td>
<td>5 (13.5)</td>
<td>0 (0)</td>
<td>32 (86.5)</td>
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<td>Ciprofloxacin</td>
<td>24 (64.9)</td>
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<td>10 (27.0)</td>
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<td>Kanamycin</td>
<td>27 (73.0)</td>
<td>5 (13.5)</td>
<td>5 (13.5)</td>
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<td>Gentamicin</td>
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<td>6 (16.2)</td>
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<td>Tetracycline</td>
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<td>Chloramphenicol</td>
<td>34 (91.9)</td>
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<td>Erythromycin</td>
<td>3 (8.1)</td>
<td>6 (16.2)</td>
<td>28 (75.7)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>37 (100.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>35 (94.6)</td>
<td>2 (5.4)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

https://doi.org/10.24171/j.phrp.2023.0181
gentamicin (10.8%, 4/37). In contrast, a majority of the isolates were resistant to ampicillin (86.5%, 32/37), erythromycin (75.7%, 28/37), and tetracycline (73.0%, 27/37).

The multidrug resistance profile of 37 *V. parahaemolyticus* strains was also examined (Table 2). All 37 *V. parahaemolyticus* isolates were found to be resistant to at least 1 antibiotic, among which 2 (5.4%), 10 (27.0%), 16 (43.2%), 7 (18.9%), and 2 (5.4%) isolates were resistant to 1, 2, 3, 4, and 5 antibiotics, respectively. Overall, the MAR index ranged from 0.11 to 0.55 (Table 2), with 18 different resistance patterns. The highest frequencies of multidrug-resistant phenotypes were observed for ampicillin, tetracycline, and erythromycin (AMP TCN ERY) with 24.32%. Additionally, 10.81% and 8.1% of isolates exhibited the (AMP TCN) and (AMP GEN TCN ERY) as well as (AMP CIP TCN ERY) phenotypes, respectively.

**ARGs in *V. parahaemolyticus* Isolates**

The prevalence of 8 ARGs in *V. parahaemolyticus* isolates was examined. As shown in Table 4, *aac(3)-IV, qnrA, and tetA* were detected in 16.2%, 27.0%, and 62.2% of isolates, whereas *blaTEM, blaOXA, blaSHV, ermA, and ermB* were undetected in all isolates tested. Six isolates were *aac(3)-IV*-positive, 4 were susceptible to gentamicin, and 2 had intermediate resistance to gentamicin. All 10 *qnrA*-positive isolates were resistant to ciprofloxacin. Of 27 isolates exhibiting resistance to tetracycline, 23 isolates harbored the *tetA* gene.

**Biofilm Formation of *V. parahaemolyticus* Isolates**

The ability of *V. parahaemolyticus* isolates to form biofilms was investigated, as depicted in Figure 2. Out of the isolates tested, 31 (83.8%) were capable of producing biofilms, whereas 6 (16.2%) could not form biofilms. Within the group of 25 multidrug-resistant isolates, 4 were identified as strong biofilm producers, adhering robustly to polystyrene. Additionally, 11 isolates were classified as moderate biofilm producers, 9 as weak biofilm producers, and only 1 isolate (VA37) was unable to produce biofilm. Conversely, among the 12 non-multidrug-resistant isolates, a single isolate demonstrated moderate biofilm formation, 6 were weak biofilm producers, and 5 were incapable of forming biofilms.

**Discussion**

*V. parahaemolyticus* is an opportunistic pathogen and a major causative agent of food-borne illnesses worldwide [7]. This bacterium causes gastroenteritis following the consumption of contaminated raw or inadequately cooked seafood. Additionally, exposure to water containing *V. parahaemolyticus* can lead to wound infections and septicemia, which can be life-threatening for individuals with certain medical conditions, such as diabetes or immune deficiency [7]. The wide distribution of *V. parahaemolyticus* in tropical seawater and mariculture environments in different countries has been reported [20–23] and its presence depends on the water temperature [13]. In this study, *V. parahaemolyticus* was present in 24.7% (37/150) of samples, and it was more frequently identified in summer (35.1%) than in winter (14.5%). Previous studies have also noted a higher prevalence of *V. parahaemolyticus* in summer and autumn than in winter and spring [13,24]. This finding supports the hypothesis that the presence of *V. parahaemolyticus* is associated with a seasonal pattern, with outbreaks in the warmer season and lower prevalence in the cold season [25]. Since *V. parahaemolyticus* is cold-susceptible, its growth may be inhibited at lower temperature in winter [7], while higher temperatures in summer promote *V. parahaemolyticus* growth with a shorter generation time and a faster growth rate, leading to a higher density of *V. parahaemolyticus* in samples [26].

It is well known that not all *V. parahaemolyticus* strains cause diseases in aquatic animals and humans, but strains harboring the *tdh* and *trh* genes produce hemolysin factors that induce inflammatory gastroenteritis, and these genes are considered as virulent indicators of pathogenic

### Table 4. The presence of antibiotic resistance genes in *Vibrio parahaemolyticus* isolates and the correlation between genotype and phenotype

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Resistance genes</th>
<th>No. of positive isolates (%)</th>
<th>Correlation between genotype and phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (n = 37)</td>
<td><em>bla</em>&lt;sub&gt;TEM&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;OXA&lt;/sub&gt;, <em>bla</em>&lt;sub&gt;SHV&lt;/sub&gt;, <em>ermA</em>, <em>ermB</em></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gentamicin (n = 37)</td>
<td><em>aac</em>(3)-IV</td>
<td>6 (16.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ciprofloxacin (n = 37)</td>
<td><em>qnrA</em></td>
<td>10 (27.0)</td>
<td>10/10</td>
</tr>
<tr>
<td>Tetracycline (n = 37)</td>
<td><em>tetA</em></td>
<td>23 (62.2)</td>
<td>23/27</td>
</tr>
<tr>
<td>Erythromycin (n = 37)</td>
<td><em>ermB</em>, <em>ermA</em></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
strains [5]. Therefore, detecting these genes in isolates is crucial for mitigating potential risks to human health. In this study, we identified a significantly higher prevalence of the *trh* gene (21.6%) than the *tdh* gene (8.1%) among 37 *V. parahaemolyticus* isolates. This pattern aligns with previous research, which found the *trh* and *tdh* genes in 15.9% and 6.1% of seawater isolates from Korea [25] and in 19.8% and 99% of isolates from seafood in China [27], respectively. In contrast, the *tdh* gene was detected at a higher level (48%) than the *trh* gene (8.3%) in an estuarine system in South Carolina, the United States in a study by Gutierrez et al. [28] in 2013. Similarly, *tdh*-positive *V. parahaemolyticus* was also more prevalent than *trh*-positive bacteria in oyster environments in Taiwan [29] and in coastal water in Saudi Arabia [30]. The differences in the distribution of *tdh*/*trh* *V. parahaemolyticus* strains are possibly due to the sampling techniques, the geographical origin, seasonal effects and the method of their detection [12]. For example, Parveen et al. [31] reported that real-time PCR could help increase the detection of *tdh*– and *trh*–positive *V. parahaemolyticus* to 13% and 40%, respectively, for water samples compared to the conventional techniques.

The antimicrobial resistance of *Vibrio* species has emerged as one of the most significant threats to fish farming, food safety, and public health [32]. Therefore, monitoring the antimicrobial susceptibility of *V. parahaemolyticus* is very important for evaluating its potential effects on environmental and human health. Our results indicated that more than 72% of *V. parahaemolyticus* isolates from mariculture were resistant to ampicillin, erythromycin, and tetracycline. These findings broadly support the work of other studies, in which 86% to 100% of *V. parahaemolyticus* strains isolated from marine environments [33,34] were resistant to ampicillin and tetracycline, and 42% to 48.3% of isolates from shrimp mariculture were resistant to erythromycin [12,35]. This high frequency of resistance confirms that these antimicrobials are widely used and becoming less effective against *V. parahaemolyticus*, likely due to the extensive use of antibiotics in the areas studied. In Vietnam, 64% of fish farms reported using at least 1 antibiotic for disease treatment and prevention, with 10% to 21% of farms utilizing tetracycline [36]. Additionally, significant levels of antibiotic residues, including ampicillin, erythromycin, and tetracycline, have been detected in aquaculture water in Vietnam, which may contribute to the increasing resistance rates to these antimicrobials [37]. Conversely, other studies have shown that ampicillin, tetracycline, and erythromycin were effective against *V. parahaemolyticus* strains, with over 90% of isolates being inhibited [10,38–40]. The variability in resistance of *Vibrio* to antibiotics may be attributed to geographical differences or variations in testing methodologies [5].

Significantly, all tested *V. parahaemolyticus* isolates were found to be susceptible to third-generation cephalosporins (ceftazidime and cefotaxime) and chloramphenicol. However, 2 isolates showed intermediate resistance to cefotaxime, and 3 isolates exhibited intermediate resistance to chloramphenicol. These findings are consistent with those of previous studies [12,13,39,41], which reported that over 90% of *V. parahaemolyticus* strains were susceptible to these antibiotics. Therefore, these drugs are considered the most
effective antimicrobials for treating *V. parahaemolyticus* infections in the areas studied.

Ciprofloxacin, a fluoroquinolone antibiotic, has been broadly employed as an alternative treatment for tetracycline-resistant bacteria [33], while aminoglycosides (kanamycin and gentamicin) antibiotics are commonly used in aquaculture production and show substantial effectiveness against a broad spectrum of bacteria [42]. However, the use of these antibiotics for the prevention and treatment of *V. parahaemolyticus* may not be effective, as the rate of antibiotic resistance among 37 *V. parahaemolyticus* isolates in the current study ranged from 13.5% to 27.0%. Comparable resistance rates have been observed in previous studies, with 9.5% to 39.8% of *V. parahaemolyticus* strains showing resistance to kanamycin, ciprofloxacin, and gentamicin [24,43]. The increasing resistance of *V. parahaemolyticus* to fluoroquinolones and aminoglycosides could be attributed to their extensive use in human medicine and aquaculture production, which may lead to significant public health concerns [44].

MAR indexing has been recognized as an efficient and cost-effective method for tracking the sources of bacterial contamination. Letchumanan et al. [5] suggest that MAR index values greater than 0.2 indicate high-risk sources of antibiotic contamination, posing a potential threat to human health. Regrettably, our study revealed that 67.57% of *V. parahaemolyticus* isolates had a MAR index exceeding 0.2, indicating a multidrug resistance phenotype. Our findings suggest that these *V. parahaemolyticus* strains were recovered from sources where antibiotics are frequently used, which could be hazardous. This is in contrast to the findings of Mok et al. [13], who reported that only 19% of *V. parahaemolyticus* strains isolated from the Korean coast were resistant to 3 antibiotics. Nonetheless, our results are in general agreement with previous observations [6,24,45], which showed that more than half of the environmental *V. parahaemolyticus* isolates exhibited multidrug resistance. Furthermore, the MAR index values in this study ranged from 0.11 to 0.55, which are significantly lower than those reported by Ahmed et al. [46], who found all *V. parahaemolyticus* isolates were resistant to at least 7 antimicrobial agents. The variation in the MAR index values may be attributed to differences in sample sources, geographic distribution, the number and types of antibiotics tested, and the methodologies employed [47].

The presence of ARGs is the basis for bacterial resistance, and a high detection rate of ARGs indicates an elevated risk of ARG transmission and a significant potential for bacteria to develop resistance [48]. In the present study, the *qnrA* gene was detected in all 10 ciprofloxacin-resistant isolates, demonstrating a strong correlation between phenotypic resistance and genotype (the presence of *qnrA*). This result aligns with previous research [44,45], in which all fluoroquinolone-resistant isolates carried the *qnrA* and *qnrS* genes. Conversely, Jeamsripong et al. [49] showed that 77.8% of 594 *V. parahaemolyticus* isolates harbored the *qnr* gene, but all isolates were susceptible to ciprofloxacin. This difference is possibly due to the evolution, mutation, and silencing of resistance genes [50] or unknown mechanisms that remain to be elucidated.

Similarly, a strong relationship between the tetracycline resistance phenotypes of *V. parahaemolyticus* isolates and the presence of ARG was also determined, as *tetA* was detected in 23 of 27 isolates that exhibited resistance to tetracycline. The overall level was found to be much higher than reported in other studies [45,51] in which the detection rate of *tetA* was 28% to 30% of tetracycline-resistant isolates. Meanwhile, *tetA* was undetected in all *V. parahaemolyticus* strains that exhibited resistance to tetracycline [5,52]. A possible explanation for this difference might be that *V. parahaemolyticus* strains used other ARGs (*tetM* and *tetS*) against tetracycline [53].

Interestingly, a high percentage of isolates showed resistance to ampicillin and/or erythromycin; however, none of the 32 or 28 isolates resistant to ampicillin/erythromycin carried the genes for macrolide or β-lactam antibiotic resistance. This discrepancy between the resistance phenotypes and genotypes for ampicillin and/or erythromycin has been noted in previous studies [5,12,45]. This may occur because resistance phenotypes can be governed by various ARGs and mechanisms [12]. Another potential explanation is that the resistance phenotypes are regulated by the efflux systems of the bacterial cells [54].

The *aac(3)-IV* gene was detected in susceptible and intermediate-resistance isolates, but not in gentamicin-resistant strains. Similarly, Beshiru and Igbinosa [45] only found the *aac(3)-IV* gene in 2 intermediate gentamicin-resistant isolates. However, the findings of the current study do not support the previous research [12], in which positive results were found for the *aac(3)-IV* gene in 12 of 61 isolates that exhibited resistance to gentamicin. Nevertheless, susceptible isolates harbored the *aac(3)-IV* gene, posing a potential risk for the preservation and transmission of ARGs [12].

Biofilm formation is a significant virulence factor for pathogenic bacteria during infection. Several *Vibrio* species, including *V. parahaemolyticus*, can produce biofilms, enabling the bacteria to establish infections and enhance their resistance to hostile environments, such as antibiotics and the host immune response [55]. This study found that more than 83% of *V. parahaemolyticus* isolates were biofilm
Strains and isolates, to isolate, is an adept swimmer [VP0950], antimicrobial resistance genes, and sources, further research is required.

Virulence genes across different marine environmental isolates, and to clarify the relationships of the genotypic variation of factors in aquatic hosts. These changes can enhance their pathogenicity or prompt genetic differentiation and adaptive variation in environmental conditions of tropical aquatic habitats may increase antimicrobial resistance, as suggested in the literature [59–61]. Moreover, the trh gene was detected in 6 biofilm-producing strains that were resistant to 4 or 5 antibiotics. This outcome is consistent with Letchumanan's findings [62], which indicated that 9 out of 13 trh-positive strains exhibited resistance to 4 or more antimicrobials. Interestingly, a correlation between biofilm phenotype and associated genes was also identified in this study, as 3 genes (VP0950, VP0952, and VP0962) that mediate biofilm formation were present in V. parahaemolyticus isolates that were identified as biofilm producers. Similar results were also reported in other studies [47,56].

The paper presents intriguing findings on the prevalence of ARGs and the virulence genes of V. parahaemolyticus strains in aquaculture environments and their implications for public health. However, this study did not assess the prevalence of V. parahaemolyticus in fish, which could pose a direct threat to human health. Nevertheless, it is widely recognized that V. parahaemolyticus is an adept swimmer capable of attaching to aquatic animals [63]. Thus, a contaminated water body may potentially infect all its fish inhabitants [64]. Alarming, some V. parahaemolyticus isolates identified in the current study carry ARGs and virulence genes, which could pose a significant risk to human health. Aquatic ecosystems, particularly, are acknowledged as hotspots for the environmental spread of pathogenic microorganisms, ARGs, and antimicrobials [1]. Furthermore, the intricate environmental conditions of tropical aquatic habitats may prompt genetic differentiation and adaptive variation in organisms. These changes can enhance their pathogenicity or lead to the emergence of new strains with distinct virulence factors in aquatic hosts [32]. Therefore, to better understand the genotypic variation of V. parahaemolyticus isolates, to determine the relatedness between clinical and environmental isolates, and to clarify the relationships of V. parahaemolyticus virulence genes across different marine environmental sources, further research is required.

Conclusion

This study provides the first insights into the prevalence, antimicrobial resistance profile, distribution of resistance genes, and virulence genes of V. parahaemolyticus strains isolated from fish mariculture environments in Vietnam. The findings suggest that rearing water is a potential source of antimicrobial-resistant V. parahaemolyticus strains, posing a risk to both aquatic animal and human health. The high frequency of resistance to several antibiotics, including ampicillin, erythromycin, and tetracycline, as well as the presence of multidrug-resistant isolates, points to the extensive use of these antibiotics in the region, which should be strictly controlled to prevent their potential spread to humans. Notably, the capacity of several multidrug-resistant isolates to produce strong biofilms indicates the persistence of V. parahaemolyticus strains in marine environments. Therefore, continuous monitoring of V. parahaemolyticus and ARGs in seafood and the mariculture environment is crucial to mitigate potential risks to human health.

Supplementary Material

Figure S1. Map of the sampling locations in Cat Ba, Hai Phong, Vietnam. The red dots indicate the places of sampling.
Table S1. List of sampling locations and number of collected samples; Table S2. List of primers used for detection of Vibrio parahaemolyticus, antimicrobial resistance genes, and virulence genes. Supplementary data are available at https://doi.org/10.24171/j.phrp.2023.0181.

Notes

Ethics Approval
Not applicable.

Conflicts of Interest
The authors have no conflicts of interest to declare.

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Availability of Data
All data generated or analyzed during this study are included in this published article (and its Supplementary Information files). For other data, these may be requested through the corresponding author.

Authors’ Contributions
Conceptualization: PVN, KCTN; Data curation: PHT, HTT, XTH; Formal analysis: KCTN; Funding acquisition: KCTN; Investigation: all authors; Writing–original draft: PVN, KCTN; Writing–review & editing: all authors. All authors read and approved the final manuscript.
References


29. Chang HC, Chen ML, Su YC, et al. Molecular characterizations of...

