
Archives of Razi Institute Journal

Volume 80, NO. 4, 2025

Journal homepage: https://archrazi.areeo.ac.ir

Original Article

Toxic Genes and Antibiotic Resistance Patterns in Vibrio Parahaemolyticus Isolates from Caught Fish of the Caspian Sea

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Article Info:

Received: 2 May 2024
Revised: 15 September 2025
Accepted: 18 September 2024

Keywords:

Vibrio parahaemolyticus, Marine Environment, Foodborne Disease, Virulence Genes.

ABSTRACT

Vibrio parahaemolyticus (*V. parahaemolyticus*) is a marine bacterium widely recognized as a predominant causative agent of bacterial foodborne outbreaks globally. The objective of our study was to determine the prevalence of toxin-producing genes and antibiotic resistance patterns in *V. parahaemolyticus* isolates obtained from fish caught in the Caspian Sea. We conducted a descriptive cross-sectional study, involving 220 fish samples from four fish species (*Rutilus kutum, Mugilidae, Cyprinus carpio* and *Perca*). Samples underwent enriched and culture for bacteriological and biochemical examination. Isolates were confirmed using the 16S rRNA flagella-specific gene of *V. parahaemolyticus* and then subjected to antimicrobial susceptibility testing using the diskdiffusion method. Additionally, PCR was employed to detect three virulence genes (*toxR*, *tdh*, and *trh* genes). Out of a total of 220 fish samples, 40 (18.2%) were found to be contaminated with *V. parahaemolyticus*. All 40 confirmed isolates possessed the *toxR* gene, and 29 (72.5%) harbored the *tdh* gene, while none of them contained the *trh* gene. The majority of the isolates were susceptible to ciprofloxacin (97.5%) and chloramphenicol (92.5%), but resistant to amoxicillin (95%) and doxycycline (95%). These findings provide valuable insights into microbial contamination of fish caught in the Caspian Sea and highlight the need for control measures due to the high prevalence of *V. parahaemolyticus* in seafood and the subsequent presence of multi drug resistance (MDR) isolates.

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How to cite this article: **Yazdanpanah E, Rajabi Z, Maftoon H, Mirbagheri SZ, Soltan Dallal Mohammad mehdi. Toxic Genes and Antibiotic Resistance Patterns in Vibrio Parahaemolyticus Isolates from Caught Fish of the Caspian Sea. *Archives of Razi Institute*. 2025;80(4):937-944.** DOI: 10.32592/ARI.2025.80.4.937

1. Introduction

The presence of pathogenic bacteria in marine environments poses concerns regarding the food safety due to their potential to cause foodborne diseases. *Vibrio parahaemolyticus* (*V. parahaemolyticus*) belongs to the Vibrionaceae family. It is a halophilic, gram-negative bacterium with a rod-shaped morphology and is capable of motility and surviving and reproducing in environments with a sodium chloride (NaCl) concentration ranging from 1 to 9%. This bacterium naturally inhabits aquatic environment such as marine, estuarine and coastal environments and is commonly associated with various types of seafood, including fish, shrimp, lobster, and shellfish (2, 3).

The *toxR* gene, thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (TRH) are some of the known virulence genes involved in Vibrio pathogenesis. Infections caused by *V. parahaemolyticus* occur due to the presence of different virulence factors including adhesins (Type I pilus), type III secretion systems (T3SS), and type VI secretion systems (T6SS). The *toxR* gene acts as a gene for *tdh* and *trh*. The *tdh* gene encodes a pore-forming protein that facilitates bacterial invasion in humans, while *trh* plays a similar role to *tdh* in causing disease (4, 5). The presence of *toxR*, *tdh*, and *trh* genes helps differentiate potentially virulent strains of *V. parahaemolyticus* from non-virulent strains. The virulence genes associated with *V. parahaemolyticus*, particularly those involved in hemolysis and cytotoxicity, cause acute gastroenteritis in the host, with symptoms such as watery diarrhea, abdominal cramps, nausea, vomiting, fever, headache, and/or bloody diarrhea in humans who consume raw, undercooked, or mishandled seafood contaminated with *V. parahaemolyticus* (6).

Additionally, contact with open wounds and *V. parahaemolyticus* can also lead to wound infections, and in rare cases life-threatening septicemia, particularly in individuals with underlying medical conditions. *V. parahaemolyticus* is responsible for numerous seafood-related food poisoning cases in many Asian countries including Japan, Taiwan and India (7, 8). In the United States, approximately 80% of the estimated 5.2 million cases of bacterial diarrhea cases are linked to foodborne illnesses (9). Given the global prevalence of *V. parahaemolyticus* gastroenteritis cases, it is crucial to investigate the prevalence of these bacteria, their virulence genes, and their impact on humans.

According to the Centers for Disease Control and Prevention report (CDC), *V. parahaemolyticus* was identified as the most common foodborne pathogen, accounting for 39–51% of Vibrio infections compared to other *Vibrio* species such as *V. vulnificus*, *V. cholerae* (non-O1 and non-O139), *V. alginolyticus*, *V.fluvialis*, *V. mimicus*, and *V. hollisae* (10).

In recent years, the emergence of antibiotic-resistant infections has become a global health concern. Therefore, timely surveillance of antibiotic-resistant bacteria and the dissemination of surveillance data are essential to address these public health issues. A significant number of *V. parahaemolyticus* strains isolated from clinical and environmental samples have shown high resistance to multiple antibiotics such as amoxicillin, ampicillin, ceftazidime, and gentamicin (11, 12). The extensive use and misuse of antibiotics for the treatment of seafood-related diseases are likely the main contributors to the rise of multiple drug resistance (MDR) in *V. parahaemolyticus* isolates (13,14). This study aims to assess the prevalence, toxin-producing genes and antimicrobial resistance patterns in *V. parahaemolyticus* isolates from Caspian Sea fish.

2. Materials and Methods

2.1 Sample Collection and Isolation of V. Parahaemolyticus

In a descriptive cross-sectional study, a total of 220 fish samples were collected from the Caspian Sea between August 2022 and August 2023. The samples consisted of four species : *Rutilus kutum*, *Mugilidae*, *Cyprinus carpio* and *Perca*. To preserve sample integrity, they were placed in sealed containers with dry ice and transported frozen to the laboratory within approximately 24 hours. Isolation of *V. parahaemolyticus* bacteria was carried out following standard protocols established by the US Food and Drug Administration (FDA). The protocols were summarized as follows:

A 5-gram portion of each sample was enriched in 45 mL of alkaline peptone water containing 3% NaCl for 24 hours. A loopful of the enriched mixture was then cultured on thiosulphate citrate bile salt sucrose (TCBS) agar (Merck, Germany). After incubation at 37°C for 24 hours, the green colonies were selected and subjected to Gram staining, oxidase activity assessment, ONPG, Triple-Sugar-Iron (TSI), Urease, Citrate, Lysin, and Arginine tests (15, 16).

2.2. Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility of the *V. parahaemolyticus* isolates was determined using the disk diffusion method, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (17).

The susceptibility tests were performed using Nutrient Agar, Muller-Hinton agar, and a panel of 10 antibiotic disks (Mast, UK) was used for antibiotic susceptibility tests, including ampicillin (10 µg), ceftazidime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), meropenem (10 µg), trimethoprim-sulfamethoxazole (25 µg), tetracycline (30 µg), amoxicillin (25 µg), and doxycycline (30 µg). *P. aeruginosa* ATCC 27853 and *V. parahaemolyticus* ATCC 17802 were used as quality control organisms.

2.3 DNA Extraction

Genomic DNA was extracted as described by the boiling method (12). Fresh colonies of the *V. parahaemolyticus* isolates were suspended in 400 µL of sterile deionized water and mixed using a vortex mixer well. The mixture was heated at 100°C for 15 minutes on a thermo block device. After heating, the samples were centrifuged at 11000 rpm for 10 minutes. The supernatants containing genomic DNAwere transferred to microtubes and stored at -20°C until further molecular analysis. The concentration of the extracted DNA was measured using a Nanodrop (Nano Drop™ One Microvolume UV-Vis Spectrophotometers).

2.4 PCR Confirmation (Detection of Virulence Genes: *tox R, tdh,* and *trh*)

PCR was performed to detect the presence of 16S rRNA, *toxR*, *tdh*, and *trh* genes in the *V. parahaemolyticus* isolates (4). The primer sequences used for the genes were as follows:

16S rRNA 16S rRNA-F: GCAGCTGATCAAAACGTTGAGT, 16S rRNA-R: ATTATCGATCGTGCCACTCAC. *toxR*-F: GTCTTCTGACGCAATCGTTG, *toxR*-R: ATACGAGTGGTTGCTGTCATG, *tdhF*: GTAAAGGTCTCTGACTTTTGGA, *tdh*-R: TGGAATAGAACCTTCATCTTCACC, *trh*-F: TTGGCTTCGATATTTTCAGTATCT, and trh-R: CATAACAAACATATGCCCATTTCCG (18,19).

PCR reactions were prepared in a total volume of 20 µL, consisting of 10 µL of Mastermix, 1.5 µL of DNA, 1 µL of primers and 7.5 µL of distilled water (D.W.).

The PCR reaction was performed using an amplification thermal cycler (Q lab, peckstar). The reaction consisted of pre-denaturation at 95°C for 5 minutes; followed by 30 cycles of the main thermal program (denaturation at 95°C for 45 seconds, annealing at 59°C for 50 seconds, extension at 72°C for 45 seconds), and a final extension at 72°C for 5 minutes. The amplified PCR products were then subjected to gel electrophoresis (Bio-Rad), and the gel image was recorded using a Gel Doc device. Positive controls included *V. parahaemolyticus* strains ATCC33847 (*toxR*+, *tdh*+) and ATCC17802 (*toxR*+, trh+), while sterile distilled water served as the negative control.

3. Results

3.1 Prevalence of *V. Parahaemolyticus* and Virulence Genes

Out of the 220 fish samples examined, 40 samples (18.2%) were found to be contaminated with *V. parahaemolyticus* (Table 1). Molecular testing confirmed the presence of *V. parahaemolyticus*, as illustrated in Figure 1. All 40 isolates of *V parahaemolyticus*, which were confirmed through biochemical testing, possessed the 16S rRNA gene.

PCR assay demonstrated that the *toxR* gene was detected in all 40 (100%) of the confirmed isolates. The presence of the thermostable direct hemolysin (*tdh*) gene was observed in 29 (72.5%) of the isolates. Notably, none of the *V. parahaemolyticus* isolates exhibited *tdh*-related hemolysin (*trh*) gene. The findings from agarose gel electrophoresis, employed for PCR amplification, are presented in Figure 2.

3.2 Antimicrobial susceptibility of the *V. parahaemolyticus* isolates

The antibiotic resistance profile of the *V. parahaemolyticus* isolates were assessed. The findings revealed high susceptibility to ciprofloxacin (97.5%), chloramphenicol (92.5%), and gentamycin (87.5%). However, resistance was noted for amoxicillin (95%), doxycycline (95%) and tetracycline (92.5%). Detailed results regarding antimicrobial resistance can be found in Table 2.

4. Discussion

The widespread consumption of fish among Iranian households, combined with the significant contribution of Caspian Sea fisheries, underscores the public health (20).

Table1. Prevalence of *Vibrio parahaemolyticus* in different species of fish samples.

Fish Sample (Species)	No. of sample	Number of positive samples	(%) of positive samples
Rutilus kutum	55	9	16.4%
Mugilidae	55	18	32.7%
Perca	55	5	9.1%
Cyprinus carpio	55	8	14.5%
Total	220	40	18.2%

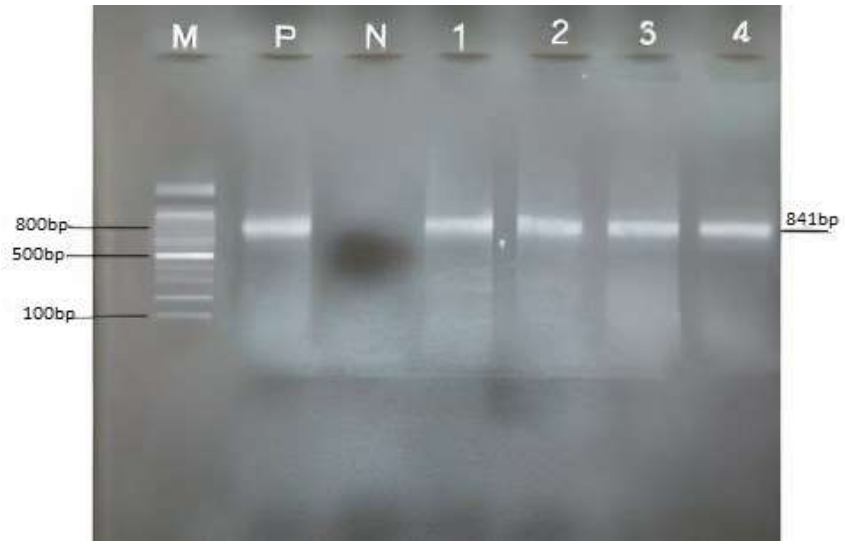

Figure 1. The presence of *V. parahaemolyticus* by PCR amplification of 16S rRNA gene. M Ladder: 100 bp; P: Positive control; N: Negative control; Numbers 1 to 4: Isolates containing 16S rRNA gene.

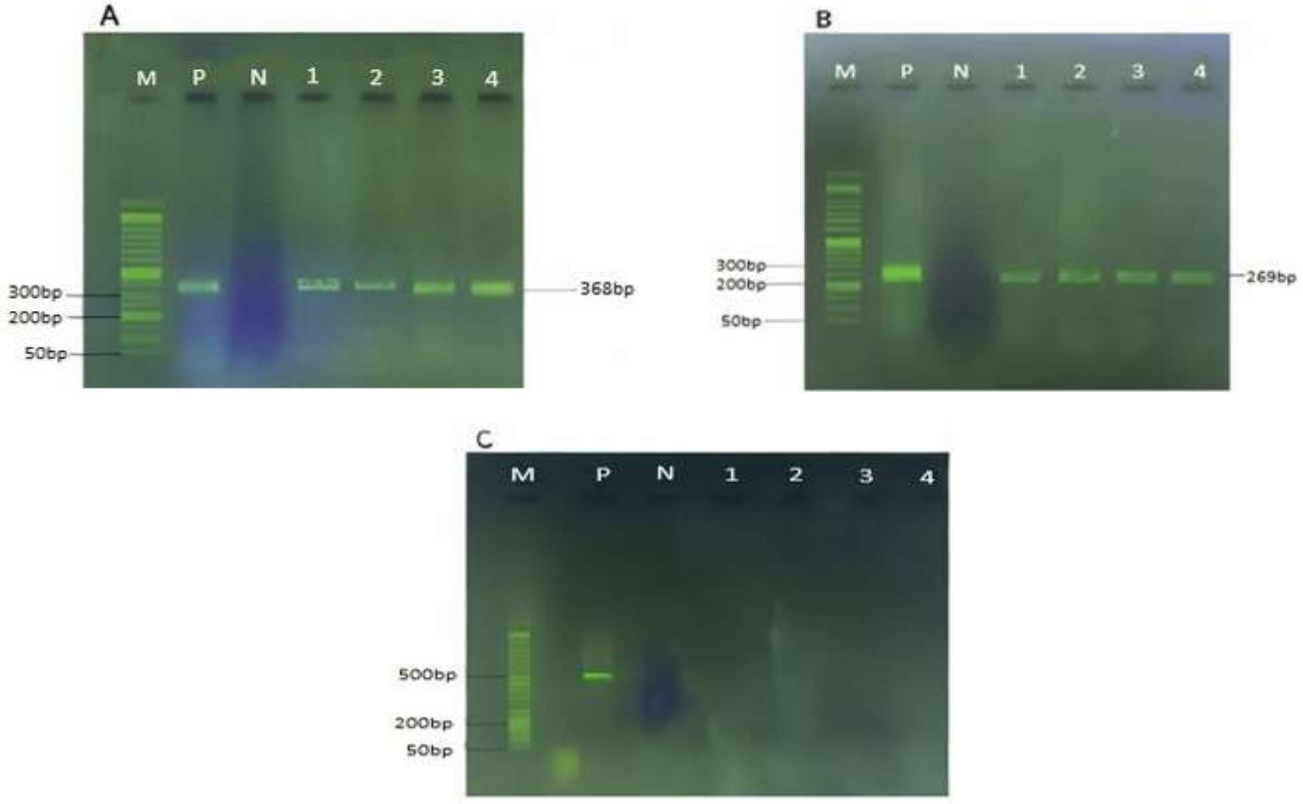

Figure 2. The presence of *V. parahaemolyticus* by detection of virulence factor genes. M: Ladder 50 bp; P: Positive control; N: Negative control. **A**, Numbers 1-4: Isolates of *V. parahaemolyticus* containing *toxR* gene (368 bp). **B**, Lines 1-4: Isolates of *V. parahaemolyticus* containing *tdh* gene (269 bp). **C**, Line 1-4: Isolates of *V. parahaemolyticus* containing *trh* gene (500 bp).

Table 2. Antimicrobial resistance profiles of *Vibrio parahaemolyticus* isolates.

Antibiotics(µg)	*V. parahaemolyticus*(n=40)			Zone diameters (mm)		
	No. (%) of Sensitive (S)	No. (%) of Intermediate (I)	No. (%) of Resistant (R)	Resistant	Intermediate	Sensitive
Ampicillin	7 (17.5)	8 (20)	25 (62.5)	13≤	16-14	17≥
Amoxicillin	(0)	2 (5)	38 (95)	13≤	17-14	18≥
Ceftazidime	3 (7.5)	5 (12.5)	32 (80)	17≤	18-20	21≥
Meropenem	1 (2.5)	8 (20)	31 (77.5)	19≤	22-20	23≥
Chloramphenicol	37 (92.5)	2 (5)	1 (2.5)	12≤	17-13	18≥
Tetracycline	(0)	3 (7.5)	37 (92.5)	11≤	14-12	15≥
Doxycycline	(0)	2 (5)	38 (95)	11≤	14-12	15≥
Ciprofloxacin	39 (97.5)	1 (2.5)	(0)	15≤	20-16	21≥
Gentamicin	35 (87.5)	2 (5)	3 (7.5)	12≤	14-13	15≥
Trimethoprim-sulfamethoxazole	33 (82.5)	6 (15)	1 (2.5)	10≤	15-11	16≥

Consequently, this study on the prevalence of *V. parahaemolyticus* holds considerable importance in the promotion of public health.

The detection of *Vibrio parahaemolyticus* in 18.1% of fish samples highlights the bacterium's prevalence in the region, likely due to its ability to tolerate high-salinity environments. These findings confirm the dominance of *V.parahaemolyticus* as the predominant microbial flora in the Caspian Sea. The majority of *V. parahaemolyticus* strains isolated from fish samples carried the *toxR* and *tdh* virulence genes, with rates of 100% and 72.5%, respectively, while the *trh* gene was absent. The absence of the *trh* gene is noteworthy, as it is associated with gastroenteritis resulting from the consumption of raw or undercooked fish and other related products.

Our results are in line with previous studies conducted nationally and internationally. For instance, Najafi (2006) and Jalali (2010) reported lower frequencies of *V. parahaemolyticus* in farmed and marine fish (5-10% and 3.9%, respectively) (14,15), suggesting a lower level of *V. parahaemolyticus* contamination in farmed fish and processed fish that have undergone proper cold storage procedures. Alipour *et al*, conducted a study on water and sediment samples from the Caspian Sea, revealing that 98 out of samples (20.3%) tested positive for *V. parahaemolyticus*, indicating a relatively high presence of the bacterium in the waters of the Caspian Sea and subsequent contamination of most fish and animals (16).

Rahimi *et al*. conducted a study on 132 shrimp and crab samples, while Raisi *et al*. examined 300 shrimp from the Persian Gulf.

They observed contamination rates of 3.03% and 9.5% with *V. parahaemolyticus*, respectively (18, 21). Another study by Safarpour *et al*. reported a presence of 22% *V. parahaemolyticus* in fish from the Persian Gulf, which is higher compared to previous similar studies. The majority of *V. parahaemolyticus* strains isolated from fish and lobster samples carried the *tdh* (23.45%) and *trh* (66.16%) virulence genes, confirming their high pathogenicity (19).

Zarei *et al*., studied the infection rates of *V. parahaemolyticus* in shrimp caught during different seasons. Their findings showed infection rates of 19% in summer, 13% in spring, 8% in autumn, and 4% in winter. The higher prevalence of *V. parahaemolyticus* in summer samples can be attributed to increased salt concentration in the water resulting from evaporation caused by heat, creating favorable conditions for bacterial growth and spread.

Additionally, 0.6% of the *V. parahaemolyticus* strains isolated from these samples carried the *toxR* virulence gene (22).

In a study conducted in Zanjan, Iran, shrimp samples were examined and found to have a 17.1% positive rate for *V. parahaemolyticus* among the 70 samples tested. Among the *V. parahemolyticus* positive samples, the *tdh* and *trh* genes were present in 2.8% and 1.4% of samples, respectively (23). Numerous studies conducted in European countries and East Asia have shown the prevalence of Vibrio species, particularly *V. parahaemolyticus*, along the coasts of Asia and East Asia (24, 25, 26).

Therefore, proper cooking of marine products is crucial in these countries to prevent gastroenteritis caused by *V. parahaemolyticus.*

In line with the aforementioned findings, Ottiviani *et al*. discovered that 11.6% of 559 oyster samples caught in the Adriatic Sea were infected with *V. parahaemolyticus*, with 7.7% of these strains carrying the *trh* gene, which contributes to hemolysis of red blood cells and weaken the immune system (24). This report indicates that *V. parahaemolyticus* poses a higher pathogenic potential due to its virulence genes when consuming contaminated raw or uncooked products. Thus, the presence of these virulence genes in *V. parahaemolyticus* contributes significantly to its pathogenicity.

The finding of Letchumanan *et al*. in Malaysia and Kang *et al*. on the coast of Korea indicated a high prevalence of *V. parahaemolyticus* species in seafood, with rates of 100% and 37.6%, respectively (25, 26). Similarly, Yang *et al*. conducted a study on 504 samples of shrimp, fish, and oysters from the southern coast of China and found that 64% of the samples were infected with *V. parahaemolyticus.* Among these samples, 8.1% and 12.2% of the strains were positive for the *tdh* and *trh* genes (27), indicating toxigenic potential. Mahmud *et al*., isolated 192 strains of *V. parahaemolyticus* from seawater and seaweeds in the K channel in Japan, and 18 samples (9.3%) carried toxic or toxigenic genes (28).

Haque *et al*., and Xiaoke *et al*., in studies conducted on fish, oysters, and shrimp in Bangladesh and China, respectively, reported frequencies of 95% and 37.7% for *V. parahemolyticus* (29, 30). Although the results of these studies were higher than our current study, none of the *V. parahaemolyticus* isolates in our study was positive for the *tdh* or *trh* genes.

Additionally, Kshirsagar *et al*., in a study on fish and shrimp samples in Gujarat, India, reported an infection rate of 11.61% for *V. parahaemolyticus* species. The *tdh* gene was found in 11.11% of the samples, but the *trh* gene was absent in all isolates, consistent with our findings (31).

The occurrence rates of contamination by various Vibrio species in marine products exhibit regional variation within Iran. This variation can be attributed to multiple factors, such as sample types, collection seasons, ecological circumstances, environmental pollution, species discrepancies, and substantial disparities in sanitary conditions from the point of fish capture to its delivery. It is noteworthy to mention that, in addition to the primary contamination stemming from fish caught in the Caspian Sea, secondary contamination can also contribute to the heightened prevalence of *V. parahaemolyticus* in fish. The lack of proper hygiene standards in fishing and processing platforms, as well as in centers for selling and distributing marine products likely plays a very significant role. Contact between the caught marine products and contaminated surfaces are likely one of the key factors leading to secondary contamination. Additionally, inadequate cooling processes for these products can further contribute to contamination.

Diversity in resistance patterns emphasizes the need for regional surveillance and tailored antibiotic strategies. The presence of multidrug-resistant *V. parahaemolyticus* in seafood poses a serious public health risk, especially when consumed raw or undercooked.

Our study found that the isolated *V. parahaemolyticus* strains demonstrated the highest resistance pattern to amoxicillin (95%) and doxycycline (95%). Other studies conducted in Iran showed that this bacterium is sensitive to chloramphenicol and cephalothin and resistant to streptomycin, ampicillin, and nalidixic acid (14). These findings are consistent with global studies of sensitivity to chloramphenicol and ciprofloxacin, and resistance to streptomycin, nalidixic acid, and ampicillin (26, 27).

The variations in antibiotic resistance patterns and the unique spectrum of resistance highlight the presence of diverse antibiotic patterns among different strains of *V. parahaemolyticus* in different regions. This underscores the significance of this species in fish contamination and the subsequent development of gastroenteritis from consuming contaminated seafood. The findings of this study underscore the relatively high microbial contamination with *V. parahaemolyticus* in fish samples caught from the Caspian Sea. Consequently, consuming these marine products, either raw or partially cooked, can pose a problem. Therefore, it is crucial to determine the antibiotic resistance pattern in these isolates to identify the most effective antibiotic and treatment approach.

In conclusion, this study provides valuable and critical information regarding the microbial contamination of fish caught from the Caspian Sea. The high prevalence of *V. parahaemolyticus* in seafood, along with the identification of multidrug-resistant isolates, presents a potential risk to human health. Therefore, appropriate control measures should be implemented to minimize the risk of

contamination. Consuming raw or undercooked fish can result in gastrointestinal issues such as heart problem, diarrhea, and gastroenteritis.

This research highlights the importance of adequately cooking marine products as the principal preventive measure against vibriosis caused by *V. parahaemolyticus*. Implementing effective health monitoring practices in fishing and distribution centers for marine products can help reduce pollution levels in these products. Furthermore, providing up-to-date information on antibiotic-resistant *V. parahaemolyticus* strains is crucial for ensuring the effective treatment of human and aquatic product infections.

Acknowledgment

We would like to extend our sincere gratitude to the Vice Chancellor for Research at Tehran University of Medical Sciences for sponsoring this research project. This article is based on a research grant approved by the Zoonoses Research Center, Tehran University of Medical Sciences.

Authors' Contribution

Study concept and design: MMSD.
Acquisition of data: MMSD, ZR, EY.
Analysis and interpretation of data: MMSD, EY.
Drafting of the manuscript: ZR, EY.
Critical revision of the manuscript for important intellectual content: MMSD, EY.
Statistical analysis: HM.
Administrative, technical, and material support: HM.

Ethics

This research was approved by the Ethics Committee of Tehran University of Medical Sciences under the code IR.TUMS.VCR.REC.1398.1069.

Conflict of Interest

The authors declare no competing interests.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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