

Prevalence and Genetic Characterization of Foodborne *Vibrio* spp. Isolated from Persian Gulf Seafood

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ABSTRACT

Background: *Vibrio* species are of particular concern due to their association with gastroenteritis after consuming raw seafood. This study aimed to assess the prevalence of *Vibrio* species and the frequency of virulence genes in *Vibrio* isolates from the Persian Gulf seafood.

Materials & Methods: In this descriptive cross-sectional study, 118 samples of marine fish, shrimp, salted fish, and salted shrimp were collected from seafood markets along the southeastern coast of Iran. The collected samples were enriched in alkaline peptone water and then cultured on thiosulfate citrate bile salt sucrose agar medium. *Vibrio* species were identified using multiplex polymerase chain reaction (PCR) targeting the *sodB*, *flaE*, and *hsp* genes. Additionally, the virulence genes *toxR*, *trh*, and *tdh* were identified through PCR.

Findings: A total of 68 *Vibrio* isolates were collected from 118 samples, including 48 (70%) *V. parahaemolyticus* isolates, 14 (20%) *V. cholera* isolates, and six (9%) *V. vulnificus* isolates. No isolates of *V. mimicus* were found. Among the isolates, the *Tdh* and *toxR* genes were detected in 16 (23%) and 35 (51%) cases, respectively. Additionally, 12 (18%) isolates contained both genes. The *Trh* gene was not detected in any of the samples.

Conclusion: The seafood market in southeastern Iran serves as a crucial hub for distributing marine products to other provinces. The presence of *Vibrio* species, particularly *V. parahaemolyticus*, along with their virulence factors in these marine products may pose a risk of infection to consumers. This underscores the importance of proper handling practices when preparing seafood.

Keywords: *Vibrio*, Sea-food, *Vibrio parahaemolyticus*, Virulence factors

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Introduction

Vibrio species are curved, Gram-negative, halophilic bacteria with over 100 species, of which 13 species are known to cause human infections ^[1]. Important species of this genus include *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* ^[2]. Since their recognition in 1950, these bacteria have caused sporadic and widespread gastroenteritis outbreaks worldwide ^[3]. *V. cholera* is a common cause of acute diarrheal disease in developed and developing countries, particularly affecting vulnerable populations lacking access to sanitary water sources. Between 2011 and 2020, a diarrhea outbreak caused by this bacterium occurred in India, resulting in 565 reported cases ^[4]. *V. vulnificus* and *V. parahaemolyticus* are also associated with gastrointestinal infections, mainly due to the consumption of contaminated food, especially raw or undercooked seafood ^[1]. *V. parahaemolyticus* produces virulence factors that are crucial for its pathogenesis, including thermostable direct hemolysin (TDH). This heat-stable, pore-forming toxin is composed of 156 amino acids and found in 48% of environmental isolates, suggesting its potential role in bacterial adaptation to various environmental conditions. The cytotoxic mechanism of TDH differs from its hemolytic activity; it increases the concentration of calcium ions in the intestinal epithelium, opens chloride channels, and enhances the secretion of chloride ions in intestinal cells ^[5]. Immunologically and genetically, TDH is similar to a heat-labile toxin known as TDH-related hemolysin (TRH). TRH functions by forming pores in the membranes of host cells, leading to cell lysis and death. This pore-forming activity disrupts cellular integrity, allowing the bacteria to invade tissues and evade the host immune response. Also, TRH enhances the overall virulence of *V. parahaemolyticus* by promoting adhesion to intestinal

epithelial cells and facilitating invasion. This is particularly associated with severe gastroenteritis cases, as strains expressing TRH tend to exhibit higher pathogenic potential compared to those that do not express TRH ^[6].

The transmembrane regulatory protein (toxR) ^[7] acts as a regulatory factor for virulence genes and biofilm formation, which are essential for the survival of *V. parahaemolyticus* in environmental conditions ^[8]. A low incidence of TDH has been found in *V. mimicus* isolates from environmental sources, suggesting that other factors may contribute to the pathogenesis of this species ^[9]. These factors include *toxR* ^[10], a *V. mimicus*-specific hemolysin (*vmh*), and a heat-stable toxin (*st*), which have been detected in *V. mimicus* ^[11].

V. parahaemolyticus could survive at temperatures of 45 to 50 °C and proliferate significantly at seawater temperatures of 14 to 19 °C. This ability helps explain the spread of this pathogen during the summer and autumn months ^[12].

Objectives: Since the southeastern Iranian fish market is one of the major seafood distribution centers across the country, and contamination of its products with foodborne pathogens has a great impact on community health, the present study was conducted to assess the prevalence of clinically-important *Vibrio* species and the frequency of *tdh*, *toxR*, and *trh* genes in *Vibrio* isolates from the Persian Gulf seafood in southeastern Iran.

Materials and Methods

Sample collection: This descriptive cross-sectional study was conducted between November 2019 and March 2020. A total of 118 seafood samples were collected from seafood markets in the southeastern port areas of the Persian Gulf in Iran using simple random sampling. The samples included shrimp (n=7), salted shrimp (n=7), pelagic

fish (n=15), benthic fish (n=83), and salted fish (n=6). All samples were promptly transferred to the laboratory in an ice box to ensure their freshness.

Enrichment and isolation: After removing the fish scales and fins, the skin was sterilized using alcohol and a flame. The muscles above the lateral line were then carefully excised. Shrimps were washed thoroughly and sterilized with alcohol and a flame, after which the carapace was aseptically removed to expose the flesh. Approximately 10 g of each sample was transferred into alkaline peptone water (Merck, Germany) and incubated at 37 °C for 6 hours. Following incubation, the samples were cultured on thiosulfate citrate bile salt sucrose agar medium (Conda Pronadisa, Spain) [13] and subsequently subjected to biochemical tests, including Gram staining, oxidase, urease, and Kligler iron agar tests [14-16]. Bacteria other than *Vibrio* species were excluded from the study. The standard strain *V. parahaemolyticus* ATCC 17802, obtained from the Iranian Genetic and Biological Resources Center, served as a positive control.

Identification of *Vibrio* species and

virulence genes: Presumptive colonies were subjected to PCR (polymerase chain reaction) testing to confirm *Vibrio* spp. Bacterial DNA was extracted by boiling method at 95 °C for 10 min [17]. *Vibrio* species were identified using multiplex PCR targeting *sodB* for *V. cholerae* and *V. mimicus*, *flaE* for *V. parahaemolyticus*, and *hsp* for *V. vulnificus* [16]. Multiplex PCR was performed in a final volume of 25 µL, consisting of 12.5 µL of Master Mix (SinaClon, Iran) containing 2U Taq polymerase 10X PCR buffer, 1.5 mM MgCl₂, 1 mM dNTP, 0.5 µL of each 10 pmol primer pair (Table 1), 3 µL of template DNA, and 7.5 µL of distilled water. PCR amplification reactions were performed on a thermal cycler (SensoQuest lab thermocycler, Germany) under the following conditions: an initial denaturation step at 95 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 59 °C for 30 s, and extension at 72 °C for 50 s; and a final extension step at 72 °C for 5 min. Eventually, PCR products were detected using 1.8% agarose gel electrophoresis, visualized with safe stain (SinaClon, Iran), and illuminated with UV

Table 1) Primer sequences used to detect *Vibrio* species and their virulence genes

Variable	Primers	Primer Sequences	Amplicon Size (bp)	Annealing Temperature	Reference
Virulence genes	<i>tdh-F</i>	5-CCA CTA CCA CTC TCA TAT GC-3	251	58 °C	(14)
	<i>tdh-R</i>	5 -GGTACTAAATGGCTGACATC-3			
	<i>trh-F</i>	5-GGC TCA AAA TGG TTA AGC G-3	250		
	<i>trh-R</i>	5-CAT TTC CGC TCT CAT ATG C-3			
	<i>toxR-F</i>	5- GTC TTC TGA CGC AAT CGT TG-3	368		
	<i>toxR-R</i>	5 -ATA CGA GTG GTT GCT GTC ATG-3			
<i>V. cholerae</i>	<i>Vc.sodB-F</i>	5- AAGACCTCAACTGGCGGTA-3	248	59 °C	(16)
	<i>Vc.sodB-R</i>	5- GAAGTGTTAGTGATCGCCAGAGT-3			
<i>V. mimicus</i>	<i>Vm.sodB-F</i>	5- GAAG CAGCACTCACCGAT-3	382		
	<i>Vm.sodB-R</i>	5- GGTGAAGACTCATCAGCA-3			
<i>V. parahaemolyticus</i>	<i>Vp.flaE-79F</i>	5- GCAGCTGATCAAAACGTTGAGT-3	894		
	<i>Vp.flaE-934R</i>	5- ATTATCGATCGTGCCACTCAC-3			
<i>V. vulnificus</i>	<i>Vv.hsp-326F</i>	5-GTCTTAAAGCGGTTGCTGC3-	410		
	<i>Vv.hsp-697R</i>	5- CGCTTCAAGTGCTGGTAGAAG-3			

(ultraviolet) light. Additionally, *toxR*, *trh*, and *tdh* virulence genes were identified by PCR under the following conditions: an initial denaturation at 95 °C for 5 min; followed by 20 cycles of denaturation at 94 °C for 1 min, primer annealing at 58 °C for 1.5 min, and extension at 72 °C for 1.5 min; and a final extension at 72 °C for 7 min ^[14].

Findings

Sample collection: Overall, 118 seafood samples were collected, including shrimp (n=7), salted shrimp (n=7), pelagic fish (n=15), benthic fish (n=83), and salted fish (n=6). A total of 74 isolates were collected from 118 samples (63%), including 48 smooth and green colonies (sucrose negative) and 26 smooth and yellow (sucrose positive) colonies. A total of 58% (68 of 118) of the samples were contaminated with *Vibrio* species. The contamination rates of seafood samples with *Vibrio* species were as follows: benthic fish 84% (57 of 68), pelagic fish 13% (9 of 68), and shrimp 3% (2 of 68). Neither salted fish nor salted shrimp showed

Table 2) Prevalence of *Vibrio* species and their virulence genes in seafood samples

Sample/ Gene	tdh	toxR	sodB (Vc*)	sodB (Vm*)	flaE (Vp*)	Hsp (Vv*)
Shrimp: n=7	1	2	0	0	2	0
Salted shrimp: n=7	0	0	0	0	0	0
Plagic fish: n=15	4	5	3	0	6	0
Bentic fish: n=83	11	28	11	0	40	6
Salted fish: n=6	0	0	0	0	0	0
Total: 118	16	35	14	0	48	6

*Vc: *V. cholera*, Vm: *V. mimicus*, Vp: *V. parahaemolyticus*, Vv: *V. vulnificus*

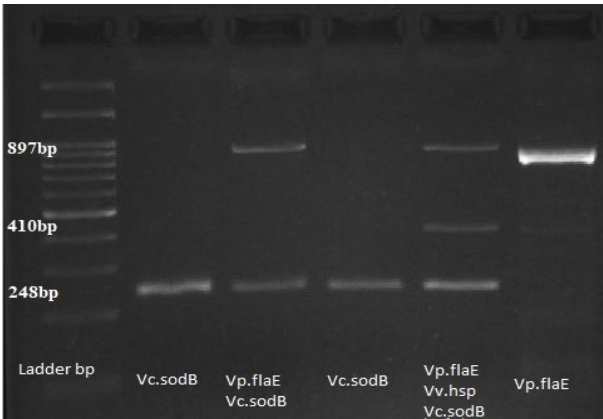


Figure 1) Detection of genes encoding *Vibrio* species using multiplex PCR targeting *sodB* for *V. cholerae* and *V. mimicus*, *flaE* for *V. parahaemolyticus*, and *hsp* for *V. vulnificus*. Line 1: 100 bp DNA ladder, line 2 and line 4: *VcsodB* (248 bp), line 3: *VpflaE* (897 bp) and *vcsodB* (248 bp), line 5: *Vvhsp* (410 bp), *vcsodB* (248 bp), and *vp flaE* (894 bp), line 6: *VpflaE* (894 bp)

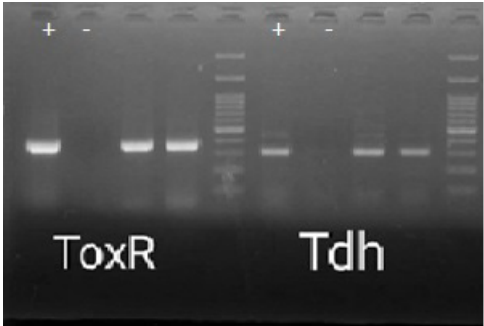


Figure 2) Detection of virulence genes *toxR* (368 bp) and *tdh* (251 bp)

any contamination with *Vibrio* species (0%) (Table 2).

Molecular identification: Molecular identification of isolates using PCR showed that 68 (58%) isolates belonged to *Vibrio* species, including *V. parahaemolyticus* (n=48, 70%), *V. cholera* (n=14, 20%), *V. vulnificus* (n=6, 9%), and *V. mimicus* (n=0, 0%). Also, 13 samples were contaminated with both *V. cholera* and *V. parahaemolyticus*.

The *tdh* and *toxR* genes were detected in 16 (23%) and 35 (51%) isolates, respectively, and 12 (18%) isolates harbored both genes (Figure 1). In *V. parahaemolyticus* isolates, the *tdh* and *toxR* genes were found in 14 (29%) and 28 (58%) isolates, respectively, with 10 (21%) isolates carrying both genes (Figure 2). Among *V. cholerae* isolates, two (14%)

and eight (57%) isolates harbored the *tdh* and *toxR* genes, respectively, while one (7%) isolate contained both genes. In *V. vulnificus* isolates, one (17%) isolate carried the *tdh* gene, two (33%) isolates harbored the *toxR* gene, and one isolate (17%) contained both genes. The *trh* and *vmsodB* genes were not detected in any of the isolates.

Discussion

The present study highlights the prevalence of *Vibrio* species, particularly *V. parahaemolyticus*, in seafood samples collected from the southeastern port areas of the Persian Gulf in Iran. Previous studies have revealed that 10.7% [18] to 22% [19] of Persian Gulf seafood is contaminated with different *Vibrio* species. However, this study found a significant increase in contamination rate, with nearly 58% of the 118 samples tested positive for *Vibrio* spp.

In the present study, the frequency of *V. parahaemolyticus* among 68 *Vibrio* isolates was 70%, which is higher than the reported seafood contamination rate in Egypt (46%) [20]. This result is also higher than the results of studies conducted on Persian Gulf seafood contamination, ranging from 39% [21] to 41.8% [18], but lower than that reported in a Malaysian study (85.7%) [22]. According to the results, *V. cholerae* was detected in 14 (20%) isolates, while *V. vulnificus* was identified in six (9%) isolates. Zangoei-Fard and colleagues (2020) found that 10.67% of the seafood samples examined were contaminated with *Vibrio* spp. Among these contaminated samples, 41.77% tested positive for *V. parahaemolyticus*, indicating a high incidence of this bacterium. Additionally, the prevalence rates of *V. cholerae* and *V. vulnificus* were reported to be 18.98 and 13.92%, respectively [18]. In the present study, only six *V. vulnificus* isolates were detected in seafood samples, which is consistent with previous findings [18, 23].

Robert-Pillot et al. (2014) found that 34.70% of the seafood samples examined were contaminated with *Vibrio* species. Among which, 31.10% tested positive for *V. parahaemolyticus*, with a higher prevalence. Additionally, *V. vulnificus* was detected at a rate of 12.60%, followed by *V. cholerae* at 0.6%. [24]. In another study conducted by Raissy et al. (2015) in Iran, the incidence rates of various *Vibrio* species in fish and shrimp samples from the Persian Gulf were as follows: *V. vulnificus* 2.65%, *V. parahaemolyticus* 3.53%, *V. mimicus* 1.76%, and *V. harveyi* 11.50% [19].

Food poisoning caused by *Vibrio* species is linked to several virulence factors, including hemolytic activity, cytotoxicity, and intestinal toxicity. These factors could lead to serious health complications such as gastroenteritis, diarrhea, sepsis, and even death [25]. Several studies have documented the widespread presence of virulence genes in environmental isolates [26, 27]. In this study, the *tdh* and *toxR* genes were present in 16 (22%) and 35 (47%) isolates, respectively. This indicates the epidemiological importance of these virulence genes in the transmission of infections caused by *Vibrio* species. A study conducted in Brazil found that the *toxR* gene was present in all *V. parahaemolyticus* isolates, but none of them carried the *tdh* or *trh* gene [25]. In a Chinese study, 9.90, 19.80, and 3.96% of *V. parahaemolyticus* isolates were positive for the *tdh*, *trh*, and both genes, respectively [28], while in another study in Nigeria, 22 (32.8%) and 39 (58.2%) *V. parahaemolyticus* isolates harbored the *tdh* and *trh* toxin genes, respectively [29]. In agreement with previous reports [6, 25], *trh* was not detected in this study. The presence of the *tdh* and *toxR* virulence genes indicates that these isolates are potentially diarrheal isolates that have acquired the ability to infect humans, causing economic losses in the seafood industry in southern regions.

Comparison of various studies shows that sample types, fishing seasons, ecological conditions, and environmental pollution significantly influence the frequency of seafood contamination [30-31]. Since the minimum growth temperature for *Vibrio* species is 5 °C, and given that the prevalence of *Vibrio* spp. increases with increasing water surface temperature [15, 31, 32], these bacteria are expected to be isolated throughout the year in the warm weather of the Persian Gulf region.

A study indicated high levels of *V. parahaemolyticus* contamination in salted shrimp [33]. However, in the present study, no contamination was found in salted products. Previous research [34, 35] has also reported the absence of *V. parahaemolyticus* growth in salted marine products. Increasing the salt and glucose concentrations to more than 2 and 0.015%, respectively, could effectively prevent biofilm formation by *V. parahaemolyticus*. This prevention occurs through several mechanisms, including osmotic stress, disruption of cell integrity, interference with quorum sensing, and alterations in metabolic pathways [34]. In the present study, the effect of seasonal variations and water temperature on the prevalence of *Vibrio* species was not investigated. It is recommended to consider this issue in future studies.

Conclusion

The isolation of *Vibrio* spp. and virulence genes from 50% of seafood samples highlights the necessity for further research into how environmental conditions affect the spread of *Vibrio* and the expression of these virulence genes. It also emphasizes the importance of tracking and identifying pathogenic *Vibrio* clones associated with the seafood supply chain. Continuous surveillance is critical for monitoring and early detection of pathogenic *Vibrio* in

seafood products. Developing models that predict environmental conditions favorable for the growth of pathogenic *Vibrio* spp. will be instrumental in designing effective control programs to reduce the spread of these bacteria.

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