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Italian Journal of Food Safety

Italian Journal of Food Safety 2026; 15:14283
doi:10.4081/ijfs.2026.14283

The role of cortisol as a physiological stress indicator in fish contamination by *Vibrio parahaemolyticus* and its implications for human health

Rzgar Farooq Rashid

Department of Medical Laboratory Technology, Shaqlawa Technical College, Erbil Polytechnic University, Kurdistan Region, Iraq

Abstract

Increased global consumption of fish and seafood, driven by their nutritional benefits, has highlighted concerns regarding bacterial contamination, particularly by *Vibrio parahaemolyticus*. This study investigates the prevalence and associated risk factors of *V. parahaemolyticus* in fish samples collected from three cities in the Kurdistan region of Iraq. A total of 185 fish samples were collected and analyzed for *V. parahaemolyticus* contamination using enrichment and isolation protocols. The overall detection rate was 9.19%, with no significant differences observed between cities, fish age, sex, or species. However, fish exhibiting abnormal physical signs, such as loose scales, pale gills, and bulging eyes, showed significantly higher contamination rates. Seasonal trends indicated a decreasing, though not statistically significant, trend in contamination from spring to summer. The cortisol level and lymphocyte count showed significant elevation, with a decrease in red cell count and abnormal physical appearance in fish compared with normal fish. These findings highlight the role of physiological and environmental factors in *V. parahaemolyticus* contamination and emphasize the need for improved biosafety measures in freshwater fish handling and processing to mitigate public health risks.

Key words: fish meat, *Vibrio parahaemolyticus*, physiological factors, human health.

Correspondence: Rzgar Farooq Rashid, Department of Medical Laboratory Technology, Shaqlawa Technical College, Erbil Polytechnic University, Kurdistan Region, Iraq.
Tel.: +9647504934560. E-mail: rzgar.faruq@knu.edu.iq

Introduction

In recent years, the global consumption of fish and seafood has surged due to growing awareness of their numerous health benefits and increased demand (Boyd *et al*., 2022). Fish meat is famous for its low cholesterol and fat content, as well as its rich composition of essential vitamins, minerals, polyunsaturated fatty acids, and high-quality animal protein, making it a vital component of a balanced diet (OECD and FAO, 2023; Ponnampalam *et al*., 2024).

Despite these nutritional advantages, seafood is particularly vulnerable to bacterial contamination. Filter feeders, such as mussels, are at high risk due to their ability to concentrate microorganisms, including bacteria and viruses, from their aquatic environments (Parlapani *et al*., 2023; Roy *et al*., 2024). Among the common pathogens associated with seafood is the bacterium *Vibrio parahaemolyticus*, a Gram-negative, curved rod prevalent in marine ecosystems. This pathogen is commonly found in raw, undercooked, or contaminated seafood, especially in temperate regions. It has emerged as a leading cause of foodborne illnesses globally (Dumen *et al*., 2020; Parlapani *et al*., 2023; Al-Garadi *et al*., 2024).

The pathogenicity of *V. parahaemolyticus* is attributed to its thermostable direct hemolysin (*tdh*) and thermostable-related hemolysin (*trh*) genes, which enable it to cause mild to moderate gastrointestinal infections. Although the infection is typically self-limiting, it can become severe, particularly in individuals with compromised immune systems. Alarmingly, *V. parahaemolyticus* has shown resistance to many antibiotics, complicating the treatment of severe cases (Álvarez-Contreras *et al*., 2021; Nguyen *et al*., 2024).

First identified as a foodborne pathogen in Japan in 1951, *V. parahaemolyticus* gained global recognition by the 1970s as a major cause of diarrheal diseases (Odeyemi, 2016; Wang *et al*., 2022; Almashhadany, Rashid, *et al*., 2024). Outbreaks of *V. parahaemolyticus* have been frequently reported, especially in Asia, but incidences in the United States and Europe have also been documented (Ndraha *et al*., 2022). Consumption of contaminated fish can lead to vibriosis in humans, characterized by symptoms such as diarrhea, abdominal cramps, nausea, vomiting, and fever. Proper handling, storage, and thorough cooking of seafood are essential to mitigate infection risks.

The rise of antimicrobial-resistant strains, such as the pandemic O3:K6 serotype, combined with the effects of climate change and warming seas, has amplified the public health challenges associated with *V. parahaemolyticus* (Al-mashhadany and Mayass, 2017; Silvester *et al*., 2022). These factors have contributed to the geographic expansion and increasing prevalence of this pathogen, underscoring the need for enhanced surveillance and control measures (Mishra *et al*., 2024). The global dissemination of highly virulent and antimicrobial-resistant strains of *V. parahaemolyticus* poses a significant public health concern (Silvester *et al*., 2022; Almashhadany, Zainel, *et al*., 2024). Climate change and rising river temperatures have been implicated in the increased prevalence and geographic expansion of *V. parahaemolyticus*, further exacerbating the risk of foodborne outbreaks. Fish are more sensitive to stressors than many other vertebrates (Wendelaar Bonga, 1997), making them ideal candidates for stress response assessments in different fields, such as environmental health and public health (Lemos *et al.*, 2023). Stress responses in fish include changes in whole-animal performance (*e.g.*, growth, disease resistance, and modified behavioral patterns) (Barton, 2002). Among different stress response biomarkers, cortisol has been shown to be a reliable stress indicator towards organic pollutants in fish (Zimmer *et al*., 2011).

Unlike marine environments, riverine ecosystems may present unique conditions that influence the survival and virulence of this pathogen. Moreover, little is known regarding the prevalence of pathogenic *V. parahaemolyticus* in locally consumed freshwater fish meat, highlighting a critical gap in food safety research. Enhanced monitoring of river water and fish products is crucial to mitigate public health risks associated with this emerging threat (Kim, 2024).

There is some evidence that environmental and social stressors disrupt microbial communities associated with the fish gut and skin (Webster *et al*., 2020); however, a potential role of cortisol in mediating these effects is unknown. Therefore, the aims of this study were to investigate the physiological factors (cortisol, red blood cell count, and lymphocyte counts) in fish that contribute to the contamination by *V. parahaemolyticus*, and to establish a simple protocol for isolation and identification of *V. parahaemolyticus* from fish meat, as well as to provide recommendations for mitigating fish contamination by this species.

Materials and Methods

Samples collection and preparation

The samples were collected from January 2024 to June 2024. A total of 185 samples were collected from three different cities in the Kurdistan region (Iraq) in sterile, labelled, sealed plastic bags. Information regarding each sample, such as age, sex, physical appearance, healthy status, gross lesions, and type of fish were recorded. Blood samples were collected from the caudal vein into two different tubes, one of them with an anticoagulant for blood picture, and the other one for serum collection to determine cortisol. Samples were transported to the lab in an icebox. In the lab, samples were analyzed immediately on the day of sampling according to the methodology described elsewhere (Noorlis *et al*., 2011). Briefly, 10 gm of fish meat were homogenized in 90 mL alkaline saline peptone water (ASPW) in a sterile polythene stomacher bag for 1 minute. Incubation of the first enrichment was done at 41.5±1°C for 6±1 hours, after which, 10 mL volume of the first enrichment culture (taken from the surface of the broth) was transferred to 90 ml ASPW as the second enrichment broth. Subsequently, a loopful (1 µL) from the second enriched broth was streaked onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates (Hassan *et al*., 2012; Al-Garadi *et al*., 2025).

Detection and identification of *V. parahaemolyticus*

Enrichment and selective culturing

10 g of minced fish tissue were homogenized in 90 mL of alkaline peptone water (pH 8.6) and incubated at 37°C for 24 hours to facilitate the enrichment of *Vibrio* species. Following enrichment, a 1 µL loopful of the broth was streaked onto TCBS agar plates, a selective medium for *Vibrio* species. The plates were incubated at 37°C for 24 hours (Hassan *et al*., 2012; Al-Garadi *et al.*, 2025).

Colony morphology and purification

Presumptive *V. parahaemolyticus* colonies on TCBS agar appeared round, smooth, opaque, and blue-green due to sucrose non-fermentation. To obtain pure cultures, single colonies were subcultured onto fresh TCBS agar and re-incubated under the same conditions (Vuoso *et al*., 2025).

Gram staining and microscopic examination

Bacterial smears were prepared from suspected colonies and subjected to Gram staining. *V. parahaemolyticus* isolates appeared as Gram-negative, curved or rod-shaped (comma-shaped) cells under microscopy (Dinh-Hung *et al*., 2025).

Biochemical characterization

The purified colonies were further confirmed using the following biochemical tests according to reference protocols (Zorrilla *et al*., 2003; Hassan *et al*., 2012; Kademi *et al.*, 2018).

- Catalase test: positive
- Oxidase test: positive
- Urease test: negative
- Indole production: positive
- Motility test: motile (observed *via* hanging drop method)
- Methyl red test: negative
- Voges-Proskauer test: negative
- Citrate utilization: negative
- Triple sugar iron agar: alkaline slant/acid butt, no H_2S production

Salt tolerance test

To confirm halophilic characteristics, suspected colonies were inoculated into nutrient broths containing 0%, 3%, 6%, 8%, and 10% NaCl and incubated at 37°C for 24 hours. *V. parahaemolyticus* exhibited growth in 3%, 6%, and 8% NaCl, but no growth in 0% and 10% NaCl, confirming its moderate halophilic nature (Kwok *et al*., 2024).

Blood indices and cortisol level estimation

Red blood cell count and lymphocytes count were done by a fully automated hematological analyzer at HPLC Scientific Research Laboratories, Mosul. Serum cortisol was quantified *via* a commercially available kit for enzyme-linked immunosorbent assay [Cortisol (Cort) ELISA Kit-Competitive, BioVenic, New York, USA] according to the manufacturer and a published protocol (Connell, 2012).

Statistical analysis

Data was analyzed by the SPSS package program (IBM, Armonk, NY, USA) for Windows. The Chi-square test was used to compare groups, and the Wilson method was used for confidence interval calculations at a significance level ≤0.05.

Results

The overall detection rate of *V. parahaemolyticus* in the collected samples is 9.19% (5.82-14.23%, 95% confidence interval) (Table 1). No significant differences were found between the cities where samples were collected from, or fish age, species, or gender. The highest contamination rate was observed in fish aged 1-2 years (10.7%), with younger fish (<1 year) showing the lowest rate (6.7%). Similarly, female fish showed a slightly higher contamination rate (9.5%) compared to males (8.5%). The infected fish showed clear abnormal appearance and physiological signs of an infection (Figure 1). Fish with abnormal physical appearance showed a significantly higher rate of positive cultures for *V. parahaemolyticus* (($p<0.001$) when compared to the positive cultures of normally looking fish. Likewise, fish with exophthalmos, pale or discolored gills, or loose scales showed significantly more infections with *V. parahaemolyticus* ($p<0.001$). However, there was no significant difference between the groups of abnormally looking fish ($p=0.8845$) or among the diseased groups ($p=0.6911$). The temporal distribution of *V. parahaemolyticus* infections during the study period showed a slight decrease in infections from spring to summer (Table 2). No significant differences between months were observed ($p=0.961$). Generally, the average infection percentage at any time among the sampled locations is estimated statistically to be between 5.82% and 14.23%.

The results in Table 3 show a significant increase in red blood cell count in fish that exhibit abnormal physical appearance ($4.3\pm1.3\times10^6$ cells/µL) compared with the normal fish ($1.7\pm0.9\times10^6$ cells/µL). But there was a significant increase in lymphocyte count and cortisol level in the serum of fish that exhibited abnormal physical appearance ($5.2\pm1.627\times10^6$ cells/µL), (97±0.5 ng/mL) respectively, compared with the normal fish ($4.4\pm0.631\times10^6$ cells/µL), (6±0.34 ng/mL) respectively.

Table 1. Characteristics of fish samples investigated for the infection of *Vibrio parahaemolyticus*.

	No. tested	Positive, n (%)	95% CI	p
Source				
Dukan city	65	4 (6.15)	2.45-14.84	0.547
Daquq city	60	7 (11.67)	5.79-22.22	
Taqtaq city	60	6 (10.00)	4.66-20.15	
Total	185	17 (9.19)	5.82-14.23	
Age (year)				
<1 year	60	4 (6.67)	2.64-15.97	0.707
1-2 years	75	8 (10.67)	5.53-19.70	
>2 years	50	5 (10.00)	4.35-21.36	
Total	185	17 (9.19)	5.82-14.23	
Species				
Coregonus lavaretus	75	6 (8.00)	3.72-16.37	0.644
Cyprinus carpio	110	11 (10.00)	5.68-17.02	
Total	185	17 (9.19)	5.82-14.23	
Gender				
Male	90	8 (8.89)	4.58-16.58	0.891
Female	95	9 (9.47)	5.08-17.06	
Total	185	17 (9.19)	5.82-14.23	

CI, confidence interval.

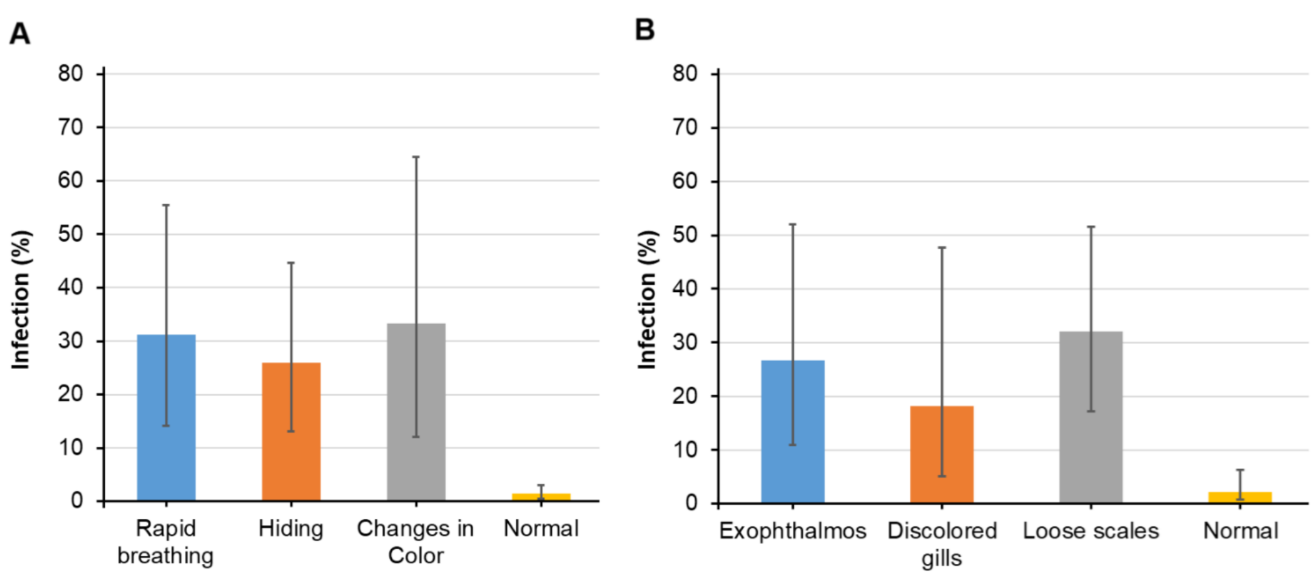

Figure 1. *Vibrio parahaemolyticus* infections in fish according to stress indicators vs. the normal appearance (**A**) and physical signs that may reflect an ongoing infection (**B**). Error bars are the 95% confidence intervals.

Discussion

Pathogenic species like *V. parahaemolyticus* are a major food-borne concern, necessitating continuous monitoring to understand their epidemiology, transmission routes, and control measures. The detection of *V. parahaemolyticus* in fish meat underscores the need for stringent biosafety measures in seafood handling and consumption. The overall contamination rate of *V. parahaemolyticus* in this work (9.2%) is slightly higher than reports from Bulgaria (6%) (Stratev *et al*., 2021) but noticeably lower than findings from regions such as Bangladesh (63.75%) (Ali *et al*., 2021) and Thailand (21.7%) (Siriphap *et al*., 2024). These regional variations could be attributed to differences in environmental conditions, sampling techniques, fish species, and detection methods (Lovell, 2017; Gavilan *et al*., 2023; Liu *et al*., 2025).

There is limited evidence directly linking the age or sex of fish to varying levels of *V. parahaemolyticus* contamination. Most studies focus on environmental factors and handling practices as primary determinants. However, fish species and physiology (*e.g*., sexual maturity, immune function, metabolic state) can affect the survival and growth of disease-causing *Vibrio* spp., as seen in shellfish (Tirsgaard *et al*., 2015; Christensen *et al.*, 2021; van Deurs *et al*., 2023; Perry *et al.*, 2024). Age and sex can influence bacterial infections in fish, though the extent varies by species and pathogen. Older fish may be more susceptible to some infections, like *Lactococcus garvieae* (Morita *et al*., 2011), while younger fish can exhibit higher mortality rates under certain conditions. Male fish have been found to be more vulnerable to acute infections, such as *Streptococcus agalactiae* (Amal *et al*., 2018), likely due to differences in immune responses. However, it is still unclear if that is the case for *V. parahaemolyticus.*

The study further identifies abnormal physical appearance, such as changes in fish coloration and breathing patterns, as factors associated with higher infection rates. Fish exhibiting loose scales, bulging eyes, or pale gills also showed low red blood cell count, increased lymphocyte count, and an increase in cortisol level, in addition to higher rates of *V. parahaemolyticus* infection. These signs likely reflect compromised health or environmental stressors, indicating potential markers for bacterial infection, as elevated cortisol is a known stress response in various fish species (Lemos *et al*., 2023). Bacterial infections in fish are often associated with symptoms like loose scales, bulging eyes, and pale gills. Loose scales can result from infections caused by bacteria, including *Vibrio* species and *Aeromonas hydrophila*, which often lead to skin lesions or ulcers (Zhou *et al*., 2024).

Stress or poor water quality can exacerbate these conditions, increasing susceptibility to infections. Bulging eyes (exophthalmia) have been observed in bacterial infections like *Streptococcus iniae* or *Pseudomonas fluorescens*, often caused by fluid accumulation due to inflammation or internal abscesses (Deng *et al*., 2024; Juárez-Cortés *et al*., 2024). Pale gills, on the other hand, are a typical sign of anemia or hypoxia, which can occur during bacterial septicemia (systemic infection) (Ruth and Denise, 2020; Faye *et al*., 2022). Pathogenic organisms, such as *Flavobacterium columnare* and *Aeromonas* spp., are known to cause damage to gill tissues in fish and other aquatic animals. This damage often results in compromised oxygen exchange due to the destruction of the delicate lamellae in the gills, leading to potentially fatal respiratory distress and a condition often referred to as gill disease or bacterial gill disease (Zamparo *et al*., 2024).

Seasonal trends showed a peak in *V. parahaemolyticus* contamination during the warmer months, correlating with elevated seawater temperatures that favor bacterial proliferation. Indeed, this observation is supported by the findings of previous studies that reported the summer season to naturally favor the growth of certain bacterial species, including vibrios, by elevating the temperature to 25-29°C (Mohi *et al*., 2010; Shen *et al*., 2017; Sheikh *et al*., 2022; Al-Garadi *et al*., 2024). This temperature-dependent growth is observed across multiple *Vibrio* species, including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (DePaola *et al*., 2003; Froelich and Daines, 2020; Randa *et al*., 2022). However, it is important to note that while temperature is a significant factor, it is not the only one influencing *Vibrio* abundance. Other factors, such as salinity, also play important roles in *Vibrio* ecology (Ahmadi *et al*., 2025).

Table 2. Isolation of *V. parahaemolyticus* according to study period.

Month	No. examined	Positive, n (%)	95% CI	p
January	35	3 (8.57)	2.97-22.42	0.961
February	30	4 (13.33)	5.29-29.64	
March	34	3 (8.82)	3.03-22.93	
April	29	3 (10.34)	3.56-26.33	
May	27	2 (7.41)	2.05-23.36	
June	30	2 (6.67)	1.86-21.37	
Total	185	17 (9.19)	5.82-14.23	

CI, confidence interval.

Table 3. Stress indicators (mean ± standard error) in all examined fish (n=185) from different sources.

Fish	RBCs *××10^6 cells/µL	Lymphocyte %	Cortisol ng/mL
Fish appear normal	4.3±1.3*	4.4±0.631	6±0.34
Fish with abnormal physical appearance	1.7±0.9	5.2±1.627*	97±0.5*

RBCs, red blood cells. *Means within the same column are significantly different at p<0.05 at each experimental time.

Conclusions

The study demonstrates that *V. parahaemolyticus* poses a measurable risk to fish consumers in the Kurdistan region, with an overall contamination rate of 9.19%. Fish with visible stress indicators, such as loose scales, pale gills, and bulging eyes, are more likely to be infected, suggesting a link between fish health and susceptibility to bacterial contamination. While no significant differences were observed between fish age, gender, or sampling locations, the study underscores the importance of maintaining proper handling and storage practices, particularly during warmer months when bacterial proliferation is likely. Enhanced surveillance, along with targeted interventions, can help mitigate the public health risks associated with *V. parahaemolyticus* in freshwater fish meat.

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Received: 26 August 2025; Accepted: 21 January 2026; Early view: 9 March 2026.

Conflict of interest: the author declares the absence of any competing interests.

Ethics approval and consent to participate: not applicable.

Availability of data and materials: data and materials are available from the corresponding author upon request.