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Research Article

Analysis of Pathogenic *Vibrio* Contamination in Marine Products along China Based on Fluorescence Quantitative PCR

Tong Wang[1,2] **Shuo Wan**[1,3] **Jian-Lian Huang**[1,3] **Zhi-Hai Sui**[4] **Cui-Juan Gao**[4] **and Yun-Guo Liu**[4]

[1]*Key Laboratory of Refrigeration and Conditioning Aquatic Products Processing, Ministry of Agriculture and Rural Affairs, Xiamen 361022, China*
[2]*College of Life Sciences and Technology, Xinjiang University, Urumqi 830046, China*
[3]*Anjoy Food Group CO. LTD., Xiamen 361022, China*
[4]*College of Life Sciences, Linyi University, Linyi 276005, China*

Correspondence should be addressed to Cui-Juan Gao; gaocuijuan@liu.edu.cn and Yun-Guo Liu; yguoliu@163.com

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At present, aquatic product pollution has become the main root of frequent food safety problems and causes economic losses. *Vibrio* is one of the main pathogens causing foodborne diseases. In this study, in order to uncover the pollution status of pathogenic *Vibrio* in the marine products of China, a total of 646 aquatic products were collected and analyzed from 10 coastal cities in China. Five kinds of pathogenic *Vibrio* were separated from these samples and monitored to explore the relationship between pollution and the pathogen. Real-time fluorescence quantitative PCR was adopted to detect foodborne *Vibrio* quantitatively in marine aquatic products. Aquatic pathogenic *Vibrio* was collected in different regions, different types of aquatic products, and different sampling places, and the difference in detection rate was statistically significant through statistical analysis. This study made a frame for the pollution degree of pathogenic *Vibrio* in marine products in China and established the dominant flora of pathogenic *Vibrio* in different types of aquatic products, which provides a theoretical basis for food safety supervision departments to take targeted prevention and control measures.

1. Introduction

Aquatic products are rich in variety and contain various nutrients required for a healthy daily diet. However, with the rapid development of modern industry and the wide use of pesticides, the aquatic animal breeding and fishing environment is deteriorating, polluting aquatic organisms, affecting the quality of aquatic products, and threatening the health of consumers [1]. The main chemical pollutants include excessive heavy metals, pesticide residues, fishery drug residues, and other organic pollutants. Careless consumption of these aquatic products will bring many health risks [2]. At present, aquatic product pollution has become the root of frequent food safety problems and causes immense economic losses [1]. Therefore, it is vital to develop rapid and reliable pathogen detection methods to reduce the frequency of outbreaks [3]. *Vibrio* is a Gram-negative rod-shaped or curved bacteria widely existing in seawater, seawater sediments, fish, shellfish, and other seafood, which includes the most common pathogenic genus in seawater and edible marine animals [4]. Eating raw or undercooked seafood can trigger foodborne diseases [5]. *Vibrio* infections can also occur as a result of skin damage (such as cuts, open wounds, or abrasions) exposed to the aquatic environment and marine animals. Due to the increasing outbreak of foodborne diseases, pathogenic *Vibrio* has become important in epidemiology [6].

Vibrio parahaemolyticus, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio fluvialis*, and *Vibrio anguillarum* are common pathogens. *V. parahaemolyticus* is a halophilic pathogen that can be found in marine, estuarine, and aquaculture environments all over the world, and is detected

constantly in aquatic products such as shellfish, crustaceans, fish, and cephalopods [7, 8]. Gastroenteritis is usually caused by eating contaminated shellfish and other raw or undercooked aquatic products containing *V. parahaemolyticus* [9]. *V. vulnificus* is another halophilic pathogen found in coastal areas and estuaries. It is pathogenic to marine animals and poses a threat to fish farming. Nevertheless, direct human infection would lead to sepsis [4]. *V. alginolyticus* is considered one of the most harmful *Vibrio*, which is pathogenic to humans and aquatic animals [10]. When exposed to polluted seawater, human soft tissues, ears and superficial wounds are vulnerable to *V. alginolyticus* [11]. Clinical symptoms include chronic diarrhea, otitis media and wound infection [12]. *V. fluvialis* is also considered a foodborne pathogen [13]. Studies have shown that *V. fluvialis* is associated with infant enterocolitis [14]. *V. anguillarum* is a severe aquatic pathogenic marine bacterium [15], causing vibriosis and sepsis in infected fish, crustaceans and bivalves [16], which caused significant economic losses [17].

Under the current situation, rapid and reliable microbial detection technology is not only an indispensable tool for government departments confronting disease outbreaks but also an effective means of disease prevention and control [18]. Therefore, it depends on accurate and specific innovative analytical methods to identify pathogenic microorganisms from clinical and food samples. Traditional bacterial identification methods are time-consuming and laborious [19]. It will take several days to confirm the presence of *Vibrio* pathogens isolated from aquatic products [3], and the diagnostic sensitivity and specificity are inadequate [20]. At the same time, traditional PCR cannot quantify the target organism directly [3, 5]. Quantitative testing is necessary to determine the intensity of infection in the sample and the prevalence of organisms in the environment. Therefore, in order to control the harmfulness caused by pathogenic *Vibrio*, a rapid, reliable and sensitive detection technique is needed to track pathogenic *Vibrio* in aquatic products. Real-time fluorescence quantitative PCR has the characteristics of rapidity, high sensitivity and high specificity, low cost and low detection limit, and does not need PCR post-processing [21]. In addition, real-time fluorescence quantitative PCR can be used to identify *Vibrio* directly, which is highly efficient for detecting and quantifying pathogenic microorganisms [5].

Real-time fluorescence quantitative PCR is a usual method to add fluorescent genes into the PCR reaction system and use the accumulation of fluorescent signals to trace the whole PCR process in real-time [22]. It quantifies the template's initial concentration of DNA or cDNA in the sample through CT value and standard curve. It can be understood as adding fluorescent substances to the PCR reaction system. The fluorescence intensity emitted by fluorochrome is directly proportional to DNA yield [23]. The intensity of the fluorescence signal in the PCR reaction system was monitored continuously [24], then the amount of specific products in pathogenic bacteria was obtained to achieve the purpose of quantitative monitoring. Real-time fluorescence quantitative PCR has a high coincidence rate and sensitivity and can monitor the amplification of each cycle [25], which is more time saving and accurate than the traditional monitoring method [26].

Aquatic products are an indispensable portion of nutrients in people's life. However, water pollution in oceans, rivers, lakes and other places has been increasingly serious in recent years. If aquatic products are cultivated from such water resources, there will also be many bacteria in these products, which will further pose a severe risk to people's health, especially the harm caused by pathogenic *Vibrio*. Quantitative PCR technology is a detection method by using SYBR Green real-time PCR to track pathogenic Vibrio in marine products quantitatively. Compared with the traditional bacterial detection method, this is safer and more convenient, which has important practical significance for monitoring pathogenic *Vibrio* in aquatic products.

In our previous study, 5 kinds of pathogenic *Vibrio* in marine products of 10 coastal cities were monitored and analyzed in 2019 to explore their possible pollution causes in order to understand the pollution status of pathogenic *Vibrio* in marine products along China, accumulate basic data for risk assessment, and provide a guide for strengthening the monitoring and prevention of foodborne diseases in China. This study aimed to quantitatively detect foodborne pathogenic *Vibrio* in aquatic products by real-time fluorescence quantitative PCR so as to provide a powerful reference for controlling the pollution of pathogenic *Vibrio* in aquatic products and reducing the foodborne diseases caused by pathogenic *Vibrio*.

2. Materials and Methods

2.1. Sample Collection. A total of 646 samples of marine aquatic products were collected in 10 cities from June to August 2019, that is Dalian, Liaoning; Yantai and Qingdao, Shandong; Lianyungang, Jiangsu; Nantong, Jiangsu;, Ningbo, Zhejiang; Wenzhou, Zhejiang; Xiamen, Fujian; Zhanjiang, Guangdong; and Beihai, Guangxi (Table 1). Among the 646 samples, 136 were collected from restaurants, 120 from barbecue shops, 117 from farmers' markets, 132 from supermarkets and 143 from online sales (Table 2). All aquatic products are stored at 4°C within 24 hours after being caught. The pollution of 5 kinds of foodborne pathogenic *Vibrio* (*V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis,* and *V. anguillarum*) was screened and monitored, repeating each process three times.

2.2. SYBR Green Real-Time PCR Detection Method. SYBR Green is a binding dye that can greatly enhance its fluorescence after binding in the small groove of double-stranded DNA. While free SYBR dye molecules will not emit any fluorescent signals, SYBR dye molecules specifically incorporate DNA double strands to emit fluorescent signals. The increase of PCR products is completely synchronized with the increase of fluorescent signals after SYBR fluorescent dye is added to the PCR reaction system. Then the whole PCR process was monitored by fluorescence signal accumulation, and the unknown concentration template was calculated quantitatively by using the standard curve. In this

Table 1: Sample collection location.

Aquatic product category	Fish	Shrimp	Shellfish	Crabs	Snails	Algae	Total
Dalian, Liaoning	12	12	10	10	13	11	68
Yantai, Shandong	11	12	10	9	10	12	64
Qingdao, Shandong	10	9	12	9	10	12	62
Lianyungang, Jiangsu	10	13	10	8	12	9	62
Nantong, Jiangsu	11	11	13	11	13	9	68
Ningbo, Zhejiang	12	10	10	12	11	11	66
Wenzhou, Zhejiang	13	12	11	12	10	13	71
Xiamen, Fujian	12	10	10	10	10	12	64
Zhanjiang, Guangdong	10	10	10	9	9	10	58
Beihai, Guangxi	11	12	12	9	10	9	63
Total	112	111	108	99	108	108	646

Table 2: Sample collection site.

Type of sampling site	Number of samples (copies)
Restaurant	134
Barbecue shop	120
Farm product market	117
Supermarket	132
Network sales	143
Total	646

experiment, specific detection primers and prepared DNA templates are designed referring to the relevant literature (Table 3). The PCR reaction procedure was set as follows: denaturation at 95°C for 5 s, annealing at 60°C for 20 s, extension at 72°C for 20 s, 40 cycles, fluorescence signals were collected at the annealing extension stage of each cycle, quantitative fluorescence detection was carried out, and the corresponding detection rate was obtained by analysis.

2.3. Statistical Analysis. SPSS 20.0 statistical software was used for data processing and analysis, and the chi-square test was applied to distinguish the difference in test results. The difference was statistically significant ($P < 0.05$).

3. Results and Analysis

3.1. Detection Level of Pathogenic Vibrio in Aquatic Products of Varied Regions. 646 samples of marine aquatic products were collected in 10 cities, includingDalian, Liaoning; Yantai and Qingdao, Shandong; Lianyungang, Jiangsu; Nantong, Jiangsu;, Ningbo, Zhejiang; Wenzhou, Zhejiang; Xiamen, Fujian; Zhanjiang, Guangdong; and Beihai, Guangxi. A total of 212 pathogenic *Vibrio* were detected, with a detection rate of 32.82%. Among them, the detection rate of pathogenic *Vibrio* in aquatic products in Zhanjiang, Guangdong is the highest, 58.62%, followed by Beihai, Guangxi (52.38%) and Xiamen, Fujian (48.44%), and Dalian, Liaoning is the lowest, 17.65%. The difference in the detection rate of pathogenic *Vibrio* in aquatic products in 10 cities is statistically significant ($P < 0.01$) through statistical analysis (Table 4).

3.2. Detection of Pathogenic Vibrio in Different Categories of Aquatic Products. Among different kinds of aquatic products, the highest detection rate is crab (46.47%), followed by shrimp (45.95%), shellfish (34.26%), snails (31.48%), fish (27.68%), and the lowest detection rate is algae (12.04%). The difference in the detection rate of pathogenic bacteria among the six kinds of aquatic products is statistically significant ($P < 0.01$) (Table 5).

3.3. Detection of Vibrio Contamination with Different Pathogenicities. As shown in Table 6, *Vibrio parahaemolyticus* has the highest detection rate of 16.1%, followed by *Vibrio alginolyticus* (7.28%) and *V. vulnificus* (4.95%), while *Vibrio anguillarum* has the lowest detection rate of 1.7%. Through statistical analysis, there was a significant difference in the detection rate of five types of pathogenic *Vibrio* in aquatic products ($P < 0.01$) (Table 6).

3.4. Detection of Pathogenic Vibrio at Different Sampling Sites. Among different sampling sites, the highest detection rate is the farm product market (67.52%), followed by network sales (39.86%), barbecue shops (30.83%), supermarkets (17.42%), and the lowest detection rate is restaurants (11.94%); Through statistical analysis, there was a significant difference in the detection rate of pathogenic *Vibrio* in aquatic product samples collected at each sampling site ($P < 0.01$) (Table 7).

4. Discussion

Taking in contaminated aquatic products would cause foodborne diseases. *Vibrio* is a serious foodborne pathogen worldwide [28]. Especially in the southeast coastal areas of China, *Vibrio* is the main pathogen causing food poisoning. This study reflects the general situation of foodborne pathogenic bacteria pollution of aquatic products in coastal cities in China. Pathogenic *Vibrio* is detected in aquatic products of different regions, different types of aquatic products, and different sampling places. Meanwhile, our research shows that the pollution rate of *V. parahaemolyticus* and *V. alginolyticus* in these aquatic products is high apparently, and they are considered the main pathogens causing foodborne diseases. When detecting *Vibrio* from fish and shellfish in Egypt, it is found that the detection rate of *V. alginolyticus* is the highest, which reflects actual local factors, such as the type and source of aquatic products,

Table 3: Oligonucleotide primer used in this study.

Strain	Prime and sequence (5′ to 3′)	Amplicon size (bp)	Reference
V. alginolyticus	Forward: ATT GAG AAC CCG ACAGAA GCG AAG Reverse: CCTAATGCGGTGATCAGTGTTACT	340	[19]
V. parahaemolyticus	Forward: GAAAGTTGAACATCATCAGCACGA Reverse: GGTCAGAATCAAACGCCG	271	[6]
V. vulnificus	Forward: TTCCAACTTCAAACCGAACTATGA Reverse: ATTCCAGTCGATGCGAATACGTTG	205	[27]
V. anguillarum	Forward: TATCACTGTTGAAGAAGGTCAAGCACTG Reverse: CGCTTCAAGTGCAGGAAGCAG	195	[17]
V. fluvialis	Forward: CGGGCTGGCCATGGACTAAACCATC Reverse: GAACCGTCATACGCGGGTTCAGAGA	280	This study

Table 4: Detection level of pathogenic *Vibrio* in aquatic products in different regions.

Region	Detected quantity (copies)	Number of samples (copies)	Detection rate (%)
Dalian, Liaoning	12	68	17.65
Yantai, Shandong	13	64	20.31
Qingdao, Shandong	12	62	19.35
Lianyungang, Jiangsu	16	62	25.81
Nantong, Jiangsu	19	68	27.94
Ningbo, Zhejiang	19	66	28.79
Wenzhou, Zhejiang	23	71	32.39
Xiamen, Fujian	31	64	48.44
Zhanjiang, Guangdong	34	58	58.62
Beihai, Guangxi	33	63	52.38
Total	212	646	32.82
χ^2 value		54.2	
P value		<0.01	

Table 5: Detection rate of pathogenic *Vibrio* in various aquatic products.

Aquatic product category	Detected quantity (copies)	Number of samples (copies)	Detection rate (%)
Fish	31	112	27.68
Shrimp	51	111	45.95
Shellfish	37	108	34.26
Crabs	46	99	46.47
Snails	34	108	31.48
Algae	13	108	12.04
Total	212	646	32.82
χ^2 value		42.9	
P value		<0.01	

Table 6: Detection of different types of pathogenic *Vibrio* in aquatic products.

Pathogenic *Vibrio* types	Detected quantity (copies)	Number of samples (copies)	Detection rate (%)
V. parahaemolyticus	104	646	16.1
V. vulnificus	32	646	4.95
V. alginolyticus	47	646	7.28
V. fluvialis	18	646	2.79
V. anguillarum	11	646	1.7
Total	212	646	32.82
χ^2 value		127.1	
P value		<0.01	

transportation and storage, treatment process of geographical source, and season [29]. Although the detection rates of *V. vulnificus*, *V. fluvialis* and *V. anguillarum* are low, studies have shown that they are highly related to extensive food poisoning and even lead to infection and death. Therefore, all these five kinds of pathogenic *Vibrio* should be paid attention to.

From the analysis of different links, this study found that the detection rate of pathogenic *Vibrio* was different in different types of aquatic products. The highest detection

Table 7: Detection levels of pathogenic *Vibrio* in different sampling sites.

Type of sampling site	Detected quantity (copies)	Number of samples (copies)	Detection rate (%)
Restaurant	16	134	11.94
Barbecue shop	37	120	30.83
Farm product market	79	117	67.52
Supermarket	23	132	17.42
Network sales	57	143	39.86
Total	212	646	32.82
χ^2 value		109.4	
P value		<0.01	

rate was found in crabs and shrimps, followed by shellfish, fish, snails and algae. The shells of shrimp and crabs may be conducive for these species to adhere, settle, and promote their survival in the aquatic environment [30]. These aquatic products are a regular diet in people's daily life, and the clean and hygienic food source is closely related to people's health and safety. Furthermore, this study demonstrates varied levels of pathogenic *Vibrio* when comparing the main pollution sources in marine products in China. For detail, in restaurants and supermarkets, the detection rate of pathogenic *Vibrio* in aquatic products is low, while in barbecue shops, farmers' markets and online sales, the detection rate is significantly high. These results suggest that the main pollution source of pathogenic *Vibrio* in coastal marine products of China is cross pollution during transportation and sales. When detecting pathogenic *Vibrio* in aquatic products in different regions, it is found that Zhanjiang, Guangdong, has the highest detection rate, followed by Beihai, Guangxi, and Shenzhen, Guangdong. With the advanced transportation service and trades between inland and coastal areas, pathogenic *Vibrio* has also spread from coastal areas into inland areas, becoming an apparent food safety risk factor that cannot be ignored in inland areas. Another problem related to *Vibrio* species is their increasing prevalence of antibiotic resistance in the marine environment, which is an important public health issue of widespread concern in various countries [31]. Particularly *Vibrio* strains with multiple antibiotic resistance may pose a serious threat to human health and the seafood industry [32,33]. Therefore, in future perspectives, we will examine the antibiotic resistance of these vibrios.

In Asian countries, including China, South Korea, and Japan, eating raw seafood is the main cause of gastrointestinal diseases. To sum up, the pollution of pathogenic *Vibrio* in aquatic products of different regions is serious. Especially, there are multiple *Vibrio* with simultaneous cross contamination in the farm product market. It is urgent to strengthen the management of all links of aquatic products and reduce pathogenic *Vibrio* pollution and prevent foodborne diseases. In addition, we recommend not eating raw or undercooked seafood in order to avoid taking in food contaminated by *Vibrio*. It would be worth suggesting adequate cooking of the seafoods before consumption to prevent future foodborne outbreaks.

This study mastered the pollution degree and source of pathogenic *Vibrio* in marine products in China and the dominant flora of pathogenic *Vibrio* in different types of aquatic products. It provides a theoretical basis for monitoring, early warning and molecular traceability of foodborne diseases. Based on the results of this study, further efforts are needed to quickly identify pathogens and control *Vibrio* contamination in aquatic products. In addition, there is an urgent need for consultation and cooperation between researchers and the government in order to minimize the risk of pathogenic *Vibrio* infection and ensure the safety of aquatic products.

Data Availability

The data collected and processed to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Tong Wang, Shuo Wan, and Jian-Lian Huang contributed equally to this work.

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