Foodborne Pathogens in Retail Oysters in South China[1]

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Objective To investigate the occurrence of important foodborne pathogens in shellstock Pacific oysters in the food markets in South China. **Methods** From July 2007 to June 2008, retail oysters were collected in different seasons from South China and analyzed for the prevalence and levels of *Listeria monocytogenes*, *Vibrio vulnificus* and *Vibrio parahaemolyticus*. **Results** None of *L. monocytogenes* could be detected in any of the 202 oyster samples tested, while *V. vulnificus* and *V. parahaemolyticus* could be detected in 67 (54.9%) and 109 (89.3%) of the 122 oyster samples analyzed, respectively, with an MPN (most probable number) value greater than or equal to 3. *V. vulnificus* and *V. parahaemolyticus* with a more than 10^2 MPN/g were found in 36 (29.5%) and 59 (48.4%) of the 122 oyster samples, respectively. The *tdh* and *trh* genes were detected in 4 (0.3%) and 8 (0.6%) of the 1 349 *V. parahaemolyticus* isolates, respectively. Of the 122 samples, 4 (3.3%) was positive for either *tdh* or *trh*. The levels of *V. vulnificus* and total *V. parahaemolyticus* in oysters in South China varied in different seasons. **Conclusion** *V. vulnificus* and pathogenic *V. parahaemolyticus* are frequently found in oysters in south China, which may pose a potential threat to public health. Data presented here will be useful for the microbiological risk assessment in oysters in China.

Key words: *Listeria monocytogenes*; *Vibrio vulnificus*; *Vibrio parahaemolyticus*; Oyster; Microbiological risk assessment

INTRODUCTION

Pacific oysters (*Crassostrea gigas*) have great capacity to pump large quantities of seawater through their bodies, and thereby bacteria in the environment will be soaked and accumulated in its body. The worldwide production of this species amounted to 4.38 million tons in 2003, which is larger than that of any other species of fish, molluscs or crustacea. Nearly 84% of the worldwide production of this species was achieved in China (http://www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en). Uncooked or partially cooked oysters are frequently consumed in China, becoming a potential vehicle of foodborne illnesses.

Vibrios are gram-negative bacteria that occur naturally in estuarine and marine environments worldwide. *Vibrio vulnificus* causes septicemia or gastroenteritis or wound infections in humans[1-3]. Infection with *V. vulnificus* usually occurs by the ingestion of contaminated shellfish (especially including raw oysters), or by contamination of preexisting wounds with seawater or shellfish. Predisposing factors, such as liver disease and compromised immune status, play an important role in the development of septicemia, while gastroenteritis and wound infections appear to be un-correlated with preexisting medical conditions. *Vibrio parahaemolyticus* is a leading cause of foodborne gastroenteritis, which is often caused by consumption of raw or insufficiently cooked shellfish[4]. Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), encoded by the *tdh* and *trh* genes, respectively, contribute to the

[1]This research was supported by grants from the Key Projects in the National Science and Technology Pillar Program of China in the Eleventh Five-year Plan Period (Contract No. 2006BAK02A15) and National Natural Science Foundation of China (Contract No. 30600499).

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0895-3988/2010
CN 11-2816/Q

pathogenicity of *V. parahaemolyticus*[5].

Listeria monocytogenes is a gram-positive bacterium found ubiquitous in the environment. Healthy people infected by *L. monocytogenes* may experience mild gastrointestinal symptoms, a flulike syndrome, or may simply become asymptomatic carriers[6]. However, individuals with a weakened immune system, pregnant women, and the elderly are at risk of developing severe sickness[7].

In the present work, the prevalence and levels of *L. monocytogenes*, *V. vulnificus* and *V. parahaemolyticus* in Pacific oysters available from the food markets in south China were addressed so as to give a quantitative risk assessment for these pathogens.

MATERIALS AND METHODS

Sample Collection

From July 2007 to June 2008, Pacific oysters were sampled in different seasons from the seafood wholesale markets or the retail markets or the restaurants in Chinese cities of Wuhan (Hubei province), Zhoushan (Zhejiang province), Fuzhou (Fujian province), Huizhou, Guangzhou, Yangjiang and Zhuhai (Guangdong province), and Beihai (Guangxi province) . The samples were transferred to the laboratory under refrigerated conditions, and subsequently analyzed on the day of sampling.

V. vulnificus and V. parahaemolyticus

The bacteriological media used herein, unless indicated, were described as in the U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual[8], and purchased from the Beijing Luqiao Co. Ltd. Vibrios were enumerated using the three-tubes most probable number (MPN) procedure. Briefly, 25 g of sample was homogenized in 225 mL of alkaline peptone water. The inoculated cultures were incubated overnight at 37 ℃. The turbid cultures were streaked onto the Cellobiose-colistin (CC) agar and the thiosulfate-citrate-bile salts-sucrose (TCBS) agar for the colony isolation of *V. vulnificus* and *V. parahaemolyticus*, respectively. The CC agar plates were incubated at 40 ℃, while the TCBS agar plates at 37 ℃ for 18 to 24 h. Three typical colonies of *V. vulnificus* or *V. parahaemolyticus* from each of the CC or TCBS plates were purified on the tryptic soy agar and tested by PCR.

The purified isolates were grown in the brain-heart infusion broth overnight at 37 ℃ and centrifuged at 10 000×g for 10 min. Cell pellets were washed twice with the sterile physiological saline, resuspended in the distilled water, and then boiled for 10 min. The bacterial lysate was used immediately for PCR or stored at -20 ℃ for use.

Primers (Table 1) specific for the *vvhA* gene was employed for the identification of *V. vulnificus*[9], while those for the R72H fragment and those for the *tdh* and *trh* genes were used for the identification of total and pathogenic *V. parahaemolyticus*, respectively[10-13].

TABLE 1

List of Oligonucleotide Primers, Target Genes, Amplicon Sizes, and Sources of Gene Sequences Used for Confirmation of *V. vulnificus* and Detection of Total and Pathogenic *V. parahaemolyticus*

Target Species	Target Gene and Primer's Sequence	Size (bp)	PCR Condition	No. of Cycles	Reference
V. vulnificus	*vvhA* Gene				
	Vvh-785F: 5′-CCG CGG TAC AGG TTG GCG CA-3′	519	94 ℃ 1 min	25	[9]
	Vvh-1303R: 5′-CGC CAC CCA CTT TCG GGC C-3′		62 ℃ 1 min		
			72 ℃ 1 min		
V. parahaemo-lyticus	R72H Fragment				
	VP33: 5′-TGC GAA TTC GAT AGG GTG TTA ACC-3′	387 or 320	94 ℃ 1 min	35	[10-11]
	VP32: 5′-CGA ATC CTT GAA CAT ACG CAG C-3′		60 ℃ 1 min		
			72 ℃ 1 min		
	tdh Gene				
	TDH-1: 5′-AGC TTC CAT CTG TCC CTT TT-3′	434	94 ℃ 1 min	30	[12]
	TDH-2: 5′-ATT ACC ACT ACC ACT CTC ATA-3′		55 ℃ 1 min		
			72 ℃ 1 min		
	trh Gene				
	R2: 5′-GGC TCA AAA TGG TTA AGC G-3′	250	94 ℃ 1 min	30	[13]
	R6: 5′-CAT TTC CGC TCT CAT ATG C-3′		55 ℃ 1 min		
			72 ℃ 1 min		

The PCR mixture in a total volume of 50 μL was comprised of 2 μL of the solution containing DNA, 1 μL of each primer stock solution (20 μmol/L), 1 μL of dNTP stock solution (containing 2.5 mmol/L of each dNTP), 0.3 μL of 5 U/L Taq polymerase (TaKaRa) and 5 μL of 10× PCR buffer. Reaction mixtures were heated at 94 ℃ for 5 min as an initial denaturation step. Amplification was then performed using the cycling conditions as specified in Table 1. All assays were terminated with a 5 min extension period at 72 ℃. Positive and negative DNA controls were included in all assays. The PCR products were electrophoresed on 2% agarose gels at 120 V, stained with ethidium bromide, and photodocumented.

L. monocytogenes

The FDA method was used to detect *L. monocytogenes*[14]. Briefly, 25 g sample was homogenized in 225 mL of buffered *Listeria* enrichment broth (BLEB) without selective agents and incubated for 4 h at 30 ℃, and then the selective agents were added and continued incubating for another 44 h at 30 ℃. The BLEB culture was streaked onto CHROMagar *Listeria* (CHROMagar microbiology, Paris, France) and incubated for 48 h at 30 ℃. At least five typical colonies of *L. monocytogenes* from CHROMagar were purified on tryptic soy agar with 0.6% yeast extract and identified by the API *Listeria* system (bioMerieux, France).

Statistical Analysis

Due to the large variation in the standard deviation of the MPN values, the median values were calculated to compare the abundance of the pathogens in samples at different seasons. Calculation was performed by using the SPSS for Windows Release 11.5 program.

RESULTS

The prevalence and levels of *V. vulnificus* and *V. parahaemolyticus* in the 122 oyster samples collected at different seasons are shown in Table 2.

TABLE 2

Prevalence and Levels of *V. vulnificus* and *V. parahaemolyticus* in Marketed Oyster Samples at Different Seasons

Vibrio Species	Season	No. of Samples Analyzed	No. of Samples Positive (%)	No. of Samples Containing the Pathogen (MPN/g)				
				3 to 10	>10 to 10^2	>10^2 to 10^3	>10^3 to 10^4	>10^4
V. vulnificus	Summer	32	20	3	7	6	4	
	Fall	30	20	5	2	6	6	1
	Winter	32	12	9	2	1		
	Spring	28	15		3	9	3	
	Total	122	67 (54.9)	17	14	22	13	1
V. parahaemolyticus	Summer	32	28	2	7	7	8	4
	Fall	30	29	12	7	2	6	2
	Winter	32	28	3	10	9	2	4
	Spring	28	24	3	6	9	3	3
	Total	122	109 (89.3)	20	30	27	19	13

V. vulnificus

V. vulnificus was detected at 3 or more than 3 MPN/g (lower limit of detection) in 54.9% (67/122) of the samples. One sample from Guangdong yielded a level of *V. vulnificus* at >10^4 MPN/g, while in 35 samples (28.7%) the counts ranged between 101 and 10^4 MPN/g. The median level of *V. vulnificus* in the samples was less than 3 MPN/g during winter, while less than 25 MPN/g in other seasons.

V. parahaemolyticus

V. parahaemolyticus was detected at 3 or more than 3 MPN/g in most (89.3%; 109/122) of the samples. About 10% (13/122) of the sample yielded the levels of the total *V. parahaemolyticus* at >10^4 MPN/g, while in 46 samples (37.7%) the counts ranged between 101 and 10^4 MPN/g. During summer and spring, the median levels of *V. parahaemolyticus* in the samples were 215 and 240 MPN/g, respectively. In contrast, during fall and winter, the median levels in oysters were 19 and 54 MPN/g, respectively.

Four (0.3%) of the 1349 *V. parahaemolyticus* strains isolated from the 122 oyster samples carried *tdh*, whereas eight (0.6%) carried *trh*. None of the isolates carried *tdh* or *trh*. Seven (5.7%) oyster samples were found to be positive for *tdh* or *trh*. Of the 122 samples analyzed, four samples were positive

for either *tdh* or *trh*, and one was positive for both *tdh* and *trh*. Of the seven samples from which pathogenic *V. parahaemolyticus* carrying *tdh* or *trh* was isolated, six (85.7%) were <10 MPN/g, and the remaining one reached 30 MPN/g.

L. monocytogenes

Of the 202 samples, none of *L. monocytogenes* was detected in 25 g of samples examined.

DISCUSSION

Occurrence of *L. monocytogenes* and Vibrios in the retail oysters collected from the food markets in South China was extensively determined in the present work.

None of *L. monocytogenes* could be detected from any oyster sample tested. Our previous study found that 2.5% of the 643 raw aquatic products contained *L. monocytogenes* (data not shown). The absence of *L. monocytogenes* in oysters observed in this study was in agreement with the previous studies[15-16], suggesting that retail oysters in South China were rarely contaminated with this pathogen.

Compared to the 54.9% prevalence of *V. vulnificus* in the market-level oysters in this study, the previously reported prevalence values more or less differed: 6% by Chan *et al*. and 9.6% to 95.2% by Cook *et al*.[17-18]. The occurrence of *V. parahaemolyticus* in the retail oysters in the present study was 89.3%, which also more or less differed with the previously reported data: 22% by Chan *et al*., 50% by Cai *et al*. and 32.3% to 96.3% by Cook *et al*.[17-19]. These differences might be due to various reasons, such as the incidence of the bacterium in the natural environment, the hygienic condition of the shellfish sample, and the method of examination.

As disclosed in the present work, *V. parahaemolyticus* at a level higher than 10^4 MPN/g was frequently found in the retail oyster samples, which was in agreement with the previous studies on the oysters in the U.S. food markets[18,20]. The regulations of food hygiene in Japan require the *V. parahaemolyticus* levels $<10^2$ MPN/g in the seafood for the raw consumption[21]. The level of concern established by FDA for *V. parahaemolyticus* in the molluscan shellfish is 10^4 per gram[22]. In China, *V. parahaemolyticus* accounts for almost one third of the bacterial foodborne outbreaks occurring in the areas covered by the National Foodborne Diseases Surveillance Network[23].

More frequent isolation of and higher levels of *V. vulnificus* could be detected in the samples collected during warmer months, which was comparable with the previous studies[24-26]. Higher levels of *V. parahaemolyticus* could be detected in the samples collected during summer and spring months in relation to the other seasons, which also confirmed the previous studies[4,27]. Indeed, infections of both *V. vulnificus* and *V. parahaemolyticus* due to the consumption of raw oysters are more common during warmer months[28].

Although *V. parahaemolyticus* is distributed in the estuarine environments worldwide, most of the environmental strains are not pathogenic to humans. Strains possessing the *tdh* or *trh* gene are highly associated with the gastroenteritis caused by this pathogen[29]. In the present study, both *tdh*- and *trh*-positive *V. parahaemolyticus* were detected in 3.3% of the samples tested, which was slightly lower than 9.6% and 6%, respectively, as reported in previous studies[30-31]. An understanding of the total and pathogenic *V. parahaemolyticus* levels in oyster is necessary for the development of control measures aiming at mitigating the risk of infection caused by this organism. However, there was greater sample-to-sample variability in the pathogenic *V. parahaemolyticus* levels than in the total *V. parahaemolyticus* ones[32]. Therefore, there is a greater uncertainty when total *V. parahaemolyticus* is used to predict the risk of illness induced by *V. parahaemolyticus*.

The minimum temperatures required for the growth of *V. vulnificus* and *V. parahaemolyticus* are 13 ℃ and 8.3 ℃, respectively[33-34].The shell oysters were generally stored at ambient temperatures without cooling devices at the food markets in China, which would lead to significant increases in the *V. vulnificus* or *V. parahaemolyticus* level.

In conclusion, the retail Pacific oysters in South China are commonly contaminated with *V. vulnificus* and *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* strains are often found in the raw retail oysters, posing a great threat to humans who consume or handle oysters. Data presented here may be used to predict the potential effect of proposed controls on exposure and as a baseline to measure the effects of implemented controls, providing useful information for the microbiological risk assessment in oysters in China.

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(*Received February* 2, 2009 *Accepted January* 14, 2010)