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Prevalence, pathogenicity, and serotypes of *Vibrio parahaemolyticus* in shrimp from Chinese retail markets

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ABSTRACT

Vibrio parahaemolyticus is one of the most common foodborne pathogens in Asian countries. *V. parahaemolyticus* contamination of retail shrimp in China has been reported previously, and such contaminated shrimp have been linked to outbreaks of foodborne diseases. However, to date, the prevalence of *V. parahaemolyticus* in retail shrimp in China has not been determined. This study aimed at identifying the prevalence of *V. parahaemolyticus* in shrimps in Chinese retail market. From May 2012 to April 2013, a total of 273 shrimp samples was obtained from retail markets in 16 provinces (19 cities) of China. *V. parahaemolyticus* was detected in 103 (37.7%) of 273 samples by the most probable number method. Bacterial densities were less than 100 MPN/g in 95.1% (98/103) of the samples. Five *trh*-positive isolates were identified from 247 isolates, and none of the isolates were *tdh*-positive. In multiplex PCR-based O-antigen serotyping of these 247 pathogenic isolates, all O-antigen serotypes, except O9, were detected, and serotype O2 was found to be the most prevalent (detected in 82 isolates). This is the first report on *V. parahaemolyticus* prevalence in retail shrimp in China, and the findings of this study can be used for microbiological risk assessment of shrimp in China.

1. Introduction

Vibrio parahaemolyticus is a gram-negative, halophilic, mesophilic, rod-shaped bacterium that is found in estuarine and marine environments all over the world (Drake, DePaola, & Jaykus, 2007; Faruque & Nair, 2006; Nishibuchi & DePaola, 2005; Oliver & Ostroff, 2001). Although *V. parahaemolyticus* is frequently present in seafood, most isolates of this species are nonpathogenic to humans (Nishibuchi & Kaper, 1995). However, isolates bearing *tdh* and *trh* are known to be pathogenic to humans. These two genes encode the haemolysins thermostable direct haemolysin (TDH) and the TDH-related haemolysin (TRH), respectively (Nishibuchi & Kaper, 1995; Zhang & Austin, 2005), which are considered major virulence factors of *V. parahaemolyticus* (Kaysner & DePaola, 2001; Okuda & Nishibuchi, 1998; Okuda, Ishibashi, Abbott, Janda, & Nishibuchi, 1997; Zhang & Austin, 2005). Therefore, at present, pathogenic *V. parahaemolyticus* isolates are identified using PCR-based methods that amplify *tdh* and *trh* gene sequences (Shirai et al., 1990; Yoh, Kawakami, Funakoshi, Okada, & Honda, 1995). However, a recent study showed that 27% of all clinical *V. parahaemolyticus* isolates did not possess *tdh* and *trh* (Jones et al., 2012), and the PCR-based identification methods failed to identify such pathogenic isolates (Honda et al., 1987; Kishishita et al., 1992; Yeung & Boor, 2004).

Serotyping has been widely used for identifying isolates in epidemiological studies. In fact, serotypes have been used extensively to distinguish between clinical isolates of *V. parahaemolyticus* (Jones et al., 2012). Furthermore, some serotypes have been reported to be highly virulent and have been identified as the causative agents of foodborne illnesses (Jones et al., 2012; Nair et al., 2007). For serotyping of *V. parahaemolyticus* isolates, traditional serological techniques that use antisera for antigen detection are commonly employed. However, traditional serotyping methods are time consuming and expensive. Therefore, for rapid and accurate identification of a wide array of *V. parahaemolyticus* isolates, a combinatorial analytical approach involving PCR-based detection and serotyping is required. To this end, a multiplex PCR-based O-antigen serotyping method was developed recently for detection and identification of *V. parahaemolyticus* (Chen et al., 2012).

V. parahaemolyticus has been isolated from a variety of seafood samples, including shrimp, oyster, fish, and clam (Liston, 1990), and it is one of the most common causative agents of gastroenteritis

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associated with consumption of raw or undercooked seafood (DePaola et al., 2003; Pan, Wang, Lee, Chien, & Horng, 1997). *V. parahaemolyticus* infections have been reported in several countries, including Japan (Kubota et al., 2008), Italy (Ottaviani et al., 2008), USA (Su & Liu, 2007), and Brazil (Leal et al., 2008). Moreover, in recent years, *V. parahaemolyticus* has been reported as one of the most common causes of foodborne diseases in China (Chen et al., 2010; Liu, Chen, Guo, & Wang, 2008).

In China, shrimp is a major fishery product. The production of shrimp and crab has grown continually in the last decade and was 5,706,000 tons in 2011 (Anonymous, 2012). Our previous studies have shown that retail foods in Chinese markets are contaminated by foodborne pathogens such as *Listeria monocytogenes* (Chen, Wu, Zhang, Yan, & Wang, 2014), *Salmonella* spp. (Chen, Zhang, Yang, Wu, & Xu, 2013), and *Campylobacter jejuni* (Zheng et al., 2014). We have previously found high prevalence of *V. parahaemolyticus* in retail shrimp in Chinese markets (unpublished data). Furthermore, shrimp contaminated with *V. parahaemolyticus* has been previously linked to outbreaks of foodborne illnesses in China (Peng, Jiang, Ruan, Liu, & Zhou, 2010). These findings underline the importance of evaluating the microbiological profile of retail shrimp for ensuring food safety. However, to date, the prevalence and levels of *V. parahaemolyticus* contamination of retail shrimp have not been determined. Therefore, in this study, we estimated the prevalence and levels of *V. parahaemolyticus* in retail shrimp in China and evaluated the virulence and serological types of the isolates.

2. Materials and methods

2.1. Sample collection

From May 2012 to April 2013, a total of 273 shrimp samples was collected from the retail markets in 16 provinces (19 cities) of China. The sampling information is shown in Table S1. The samples consisted of 111 fresh shrimp samples, 73 frozen shrimp samples, and 89 dried shrimp samples. The samples were placed in sterile sealed plastic bags, transported to the laboratory in sealed containers containing ice, and analysed within 24 h.

2.2. Enumeration of *V. parahaemolyticus*

All bacteriological media, unless otherwise indicated, were purchased from the Guangdong Huankai Co. Ltd. *V. parahaemolyticus* load in shrimp was estimated using the most probable number (MPN) method in accordance with the Bacteriological Analytical Manual standard method with some modifications (Kaysner & DePaola, 2004). Briefly, 25 g of the samples was homogenised for 60 s in stomacher bags (Huankai, Guangzhou, China) with 225 mL of alkaline peptone water (APW) containing 3% NaCl. Serial 10-fold dilutions were prepared up to 1:10^3dilution and 3 × 1 mL portions of each dilution were inoculated into 9 mL of APW with 3% NaCl. The inoculated media were incubated at 37 °C for 16–18 h. After incubation, samples were collected from the top 1 cm of each culture tubes that showed turbidity using an inoculation loop. The collected samples were streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates and incubated at 37 °C for 18–24 h. Presumptive *V. parahaemolyticus* colonies (green or blue green colonies, 2–3 mm in diameter) were selected from each plate and were streaked onto Chromogenic Vibrio Medium and incubated at 37 °C for 24 h. The mauve colonies were selected for further *V. parahaemolyticus* identification tests. These tests included oxidase activity assessment, Gram staining, 3.5% NaCl triple sugar iron (TSI) test, halophilism tests, and API 20E diagnostic strips system (Biomerieux Company, France) testing. Total numbers of *V. parahaemolyticus* in samples were determined by converting the numbers of culture tubes positive for *V. parahaemolyticus* to MPN/g using a MPN table.

2.3. PCR confirmation of total and pathogenic *V. parahaemolyticus*

Genomic DNA was extracted using a commercial Universal DNA Extraction Kit (Sangon, Shanghai, China) according to the manufacturer's instructions.

Total and pathogenic *V. parahaemolyticus* were identified by amplifying *toxR*, *tdh*, and *trh* as described previously (Kim et al., 1999; Tada et al., 1992). The primer sequences and amplicon size used in this study are shown in Table 1. All oligonucleotide primers were synthesised by Sangon Biotech (Shanghai, China). The PCR mixture (total volume 25 μL) contained 1× DreamTaq™ Green PCR Master Mix (Fermentas, USA), 0.4 μmol/L of each primer (Table S2), and 2 μL of DNA template. The reaction consisted of an initial denaturation step (94 °C for 5 min), followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min. The reaction was concluded by a final extension cycle at 72 °C for 7 min. The PCR was conducted in a Bio-Rad PTC-200 Thermal Cycler (BIO-RAD, USA). Genomic DNA of *V. parahaemolyticus* strains ATCC 33847 (*tdh*+) and ATCC 17802 (*trh*+) were used as positive controls and distilled water was used as the negative control. Both positive and negative controls were included in all assays. The amplified products were then analysed by electrophoresis in a 2% agarose gel containing Gold View (SBS Genetech, Beijing); the bands were visualised using ImageQuant 350 Capture (GE, USA).

2.4. Multiplex serotyping PCR

The serotypes of *V. parahaemolyticus* isolates were identified using the PCR-based O-antigen serotyping technique. The sequences of the primers used for this assay are shown in Table S3. The 12 primer pairs were divided into two groups to generate target DNA, and the primer concentrations and amplification conditions used were as previously described (Chen et al., 2012).

3. Results

3.1. Prevalence and density of *V. parahaemolyticus* in shrimp samples

The prevalence and density of *V. parahaemolyticus* in the 273 shrimp samples are shown in Table 1. In 273 shrimp samples, 103 samples (37.7%) were positive for *V. parahaemolyticus*. Of these, 78 samples (70.3%) were fresh shrimp, 16 samples (21.9%) were frozen shrimp, and 9 samples (10.1%) were dried shrimp.

In the majority (95.1%; 98/103) of the *V. parahaemolyticus*-positive samples, pathogen densities ranged between 3 and 100 MPN/

Table 1
Prevalence and levels of *Vibrio parahaemolyticus* in different shrimp samples obtained from Chinese retail markets.

Shrimp samples	No. of samples analysed	No. of samples positive (%)	No. of samples containing the pathogen (MPN/g)			
			3–10	>10–10^2	>10^2–10^3	>10^3
Fresh shrimp	111	78 (70.3)	44	30	1	3
Frozen shrimp	73	16 (21.9)	12	3	1	0
Dried shrimp	89	9 (10.1)	8	1	0	0
Total	273	103 (37.7)	64	34	2	3